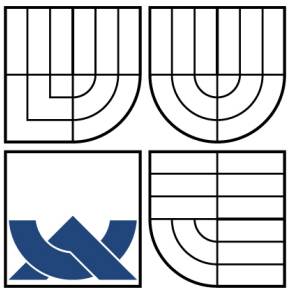

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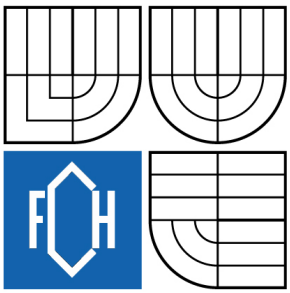

FAKULTA CHEMICKÁ
ÚSTAV CHEMIE MATERIÁLŮ

FACULTY OF CHEMISTRY
INSTITUTE OF MATERIALS SCIENCE

DETERMINATION OF COLLAGEN DEGRADATION DEGREE USING SPECTROSCOPY

URČENÍ STUPNĚ DEGRADACE KOLAGENU POMOCÍ SPEKTROSKOPIE

DIPLOMOVÁ PRÁCE
MASTER'S THESIS

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Vysoké učení technické v Brně
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Zadání diplomové práce

Číslo diplomové práce:	**FCH-DIP0321/2008**	Akademický rok: **2008/2009**
Ústav:	Ústav chemie materiálů	
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Studijní program:	Chemie, technologie a vlastnosti materiálů (N2820)	
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Vedoucí diplomové práce:	**Ing. Lucy Vojtová, Ph.D.**	
Konzultanti diplomové práce:		

Název diplomové práce:

Určení stupně degradace kolagenu pomocí spektroskopie

Zadání diplomové práce:

Vlivem některých chemikálií nebo tepelným účinkem ztrácí kolagen své původní nativní vlastnosti a denaturuje až na želatinu. Určení stupně degradace kolagenu před, během i po přípravě vzorků je důležitým faktorem pro další postupy.
Literární rešerše:
1) Vlastnosti kolagenu se zaměřením na jeho stabilitu
2) Vliv podmínek (světla, pH, teploty, rozpouštědla atd.) na stárnutí (degradaci) kolagenu
3) Metody charakterizace stupně degradace kolagenu
Experimentální práce:
1) Působení různých fyz. podmínek na přírodní kolagen
2) Stanovení stupně degradace jak pomocí UV tak i na základě FT-IR metody.
3) Vyhodnocení stárnutí kolagenu
Závěr

Termín odevzdání diplomové práce: 22.5.2009

Diplomová práce se odevzdává ve třech exemplářích na sekretariát ústavu a v elektronické formě vedoucímu diplomové práce. Toto zadání je přílohou diplomové práce.

Bc. Kateřina Fišerová Student(ka)	Ing. Lucy Vojtová, Ph.D. Vedoucí práce	prof. RNDr. Josef Jančář, CSc. Ředitel ústavu

V Brně, dne 1.10.2008

doc. Ing. Jaromír Havlica, DrSc.
Děkan fakulty

ABSTRACT

Presented diploma thesis deals with the characterization of collagen properties after exposure to various physical and chemical conditions. The main aim was to describe changes in the structure of collagen due to its modification during the preparation of samples, to assess the impact of these changes on the further use of collagen and the selection of appropriate methods for determining the collagen properties.

Chemical structure of collagen, its use as biomaterials, collagen stability, and ways to determine the degree of disruption of the native collagen structure are described in the theoretical part.

In the experimental work, water solutions based on the bovine collagen type I were treated by varying intensities of collagen disintegration (from 1 min at 6 000 rmp to 5 min at 14 000 rpm), different preparation temperatures (4 °C, lab. temp. and 30 °C), preparation time (from 1 day up to 5 days), time of light radiation effect (from 1 day up to 14 days) and different values of pH (from 4 to 8).

In order to describe the collagen structure and the changes that occurred during the modification of collagen by various conditions, prepared samples were analyzed both by UV-VIS and FT-IR spectroscopy. Either aqueous solution of 2,4,6-trinitrobenzensulfonic acid (TNBS) or ninhydrin solution in alcohol were used for comparison as the reagents with free amino groups of collagen in order to analyze the suitable colored products by UV-VIS spectroscopy. As for FT-IR spectroscopy, collagen samples were measured either in the form of KBr tablets or in films by transmission technique. To evaluate changes in the collagen structure the deconvulation method of the amide I group characteristic band between 1590 - 1720 cm^{-1} in the infrared spectrum of collagen was used. The samples morphology was observed on the collagen lyophilized sponges by scanning electron microscopy (SEM).

In accordance with theoretical assumptions, results confirmed that the largest disruption in collagen structure arise from long standing of collagen solution on the light. Significant changes cause as well increasing temperature and almost no effect on the collagen structure had disintegration intensity of collagen solution during the samples preparation. As for increasing pH, samples were stable and homogeneous until ph 5 but since the ph was higher the collagen has been separated and precipitated from water, which affected and distorted the results from UV-VIS analyses.

The samples disintegrated at 4 °C for 4 min at 14 000 rpm without changing the pH and freezed freshly after the preparation with no standing delay on the light achieved the best characteristics in comparison with untreated collagen solution.

It was found that an appropriate method for determining the degree of collagen structure disruption is UV-VIS spectroscopy using TNBS as the reagent in combination with infrared spectroscopy of collagen films.

KEY WORLDS: collagen, ageing, gelatine, lyophilization.

ABSTRAKT

Předložená diplomová práce se zabývá charakterizací vlastností kolagenu po působení různých fyzikálních a chemických podmínek. Jejím cílem je popsat změny struktury kolagenu způsobené jeho modifikací během přípravy vzorků, zhodnotit dopad těchto změn na další použití kolagenu a výběr vhodné metody pro determinaci vlastností kolagenu.

V literární rešerši je popsána chemická struktura kolagenu, jeho využití jako biomateriálu, stabilita kolagenu a způsoby určení stupně porušení nativní kolagenní struktury.

V experimentální části jsou vodné roztoky hovězího kolagenu typu I modifikovány působením různých podmínek - různé intenzity a délky dezintegrace (1 min při 6 000 ot.min^{-1} až 5 min při 14 000 ot.min^{-1}), různé teploty přípravy (4 °C, laboratorní teplota a 30 °C), různé doby přípravy (1 až 5 dní), působením světla (1 až 14 dní) a úpravou pH (4 až 8).

Za účelem popsání struktury kolagenu a změn, k nimž došlo během modifikace kolagenních vzorků působením různých podmínek byly kolagenní roztoky analyzovány UV-VIS spektroskopií a infračervenou spektroskopií s Fourierovou transformací (FT-IR). Pomocí UV-VIS byly analyzovány barevné produkty reakce volných aminoskupin přítomných v kolagenu s vhodným činidlem. Pro porovnání byl jako reagent použit buď vodný roztok kyseliny 2,4,6-trinitrobenzensulfonové (TNBS) nebo roztok ninhydrinu v alkoholu. V případě FT-IR spektroskopie byly kolagenní vzorky analyzovány buď v podobě KBr tablet nebo v podobě filmů pomocí transmisní techniky. Pro vyhodnocení změn ve struktuře kolagenu byla využita metoda dekonvulace píku amidu I, jednoho z charakteristických pásů v infračerveném spektru kolagenu, který leží v oblasti mezi 1590 - 1720 cm^{-1}. Na základě snímků pořízených pomocí rastrovací elektronové mikroskopie (SEM) byla porovnána morfologie kolagenních vzorků.

V souladu s teoretickými předpoklady bylo zjištěno, že k nejvýraznějšímu porušení kolagenní struktury dochází při dlouhodobém stání kolagenního roztoku na světle. Podstatné změny způsobuje i zvýšení teploty a téměř žádný vliv na strukturu kolagenu nemá intenzita dezintegrace kolagenního roztoku. V případě zvýšení pH, byly vzorky do pH 5 stabilní a homogenní, ale poté se vzrůstajícím pH docházelo k oddělení kolagenní fáze a srážení kolagenu, což ovlivnilo a zkreslilo výsledky z UV-VIS analýz.

Nejlepších vlastností ve srovnání s původním nemodifikovaným kolagenním roztokem dosáhly vzorky dezintegrované při 4 °C po dobu 4 min při 14 000 ot.min^{-1} beze změny pH a zamražené čerstvě po přípravě bez prodlevy stání na světle.

Na základě výsledků analýz se jako vhodné metody pro stanovení stupně porušení kolagenní struktury ukázala UV-VIS spektroskopie s využitím TNBS v kombinaci s infračervenou spektroskopií kolagenních filmů.

KLÍČOVÁ SLOVA: kolagen, stárnutí, želatina, lyofilizace

FIŠEROVÁ, K. *Determination of collagen degradation degree using spectroscopy*. Brno: Brno University of Technology, Faculty of Chemistry, 2009. 82 p. Supervisor of Diploma Thesis Ing. Lucy Vojtová, Ph.D.

DECLARATION

I declare that the diploma thesis has been worked out by myself and that all the quotations from the used literary sources are accurate and complete. The content of the diploma thesis is the property of the Faculty of Chemistry of Brno University of Technology and all commercial uses are allowed only if approved by both the supervisor and the dean of the Faculty of Chemistry, VUT.

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student's signature

Acknowledgments:
I would like to thank my promotor Ing. Lucy Vojtová, Ph.D. for many valuable comments, helpful discussions and english correction. I also thank colleagues who contributed to the successful completion of this work.

CONTENT:

1 INTRODUCTION

The term collagen applies to a broad group of proteins. They are a family of extracellular matrix proteins that play a dominant role in maintaining the structure of various tissues and also have many other important functions (for example adhesion or tissue remodelling).

At present, at least 22 different types of collagen have been outlined. The most common is type I collagen, which is present in the skin, bones, tendon, etc. Aminoacid composition of this collagen and detail in its amino acid sequence of two different polypeptidic chains is already known. As a structural proteins collagens are main organic component of the support system of animal organism, bone, cartilage, tendon, ligament and skin. In addition, collagens are an important component of blood vessel walls, basal membranes, the cornea, and some organs of the body.

Because collagen is involved in all multicellular organisms, its resources are almost unlimited. In addition to the leather industry, which serves as the main raw material in the production of leather, the collagen is used in a number of other disciplines. Pharmaceutical products, such as hemostatic felt and foam, surgical thread, collagen membranes, filters and so prepared using collagen are important. Moreover, collagen is significant component of dietary supplements in which collagen have a prophylactic and therapeutic role in diseases of key locomotive parts, namely the joints and tendons. It is also used in the cosmetics industry in products for the suppression of manifestations of aging skin. Numerous applications of collagen resulting from physiological similarities, or even conformity of the collagen with physical collagen. Collagen-based biomaterials are expected to become a useful matrix substance for various biomedical applications in the future. Collagen exhibits biodegradability, weak antigenecity and superior biocompatibility compared with other natural polymers, such as albumin and gelatin.

As well as other proteins, collagen loses its native properties when subjected to denaturation by some chemical or thermal effects. Denaturation product of collagen is gelatin. When collagen is denatured to native structure three grafts corresponding to collagen triple helix are formed. Many physical properties of the collagen are changed by hydrolytic degradation. The speed of this process depends on temperature, pH of the solution and internal pressure of the solution in lower extent.

Change of physical properties of collagen and/or collagen structure within the preparation process, however, affect the reproducibility of collagen sample preparation or cross-linking reactions. Therefore it is important to find a suitable method useful to describe changes in collagen structure that occur during the preparation of samples. The aim of this thesis is the validation of the method that is capable and sufficiently sensitive to detect changes in the collagen arising from the collagen sample preparation.

2 THEORETICAL PART

2.1 Collagen characteristics

Collagen is the major structural component of connective tissues. In vertebrates, it represents about one-third of their total protein content. At least 22 fibrillar and non-fibrillar, genetically different, types of collagen have been distinguished. Different collagen types are necessary to confer distinct biological features to the various types of connective tissues in the body (Tab. 1). The fibrillar collagen type I is the major component of tendon, bone, skin, and other tissues [1].

Tab. 1 *Chain compositon and body distribution of collagen types [2].*

Collagen type	Chain composition	Tissue distribution
I	$(\alpha1(I))_2\ \alpha2(I)$, trimer $(\alpha1(I))_3$	Skin, tendon, bone, cornea, dentin, fibrocartilage, large vessels, intestine, uterus, dermis
II	$(\alpha1(II)_3$	Hyaline cartilage, vitreous, nucleus pulposus, notochord
III	$(\alpha1(III))_3$	Large vessels, uterine wall, dermis, intestine, heart valve, gingiva (usually coexists with type I except inbone, tendon, cornea)
IV	$(\alpha1(IV)_3\ \alpha2(IV)$	Basement membranes
V	$\alpha1(V)\ \alpha2(V)(3(V)$ or $(\alpha1(V))_2\ \alpha2(V)$ or $(\alpha1(V))_3$	Cornea, placental membranes, bone, large vessels, hyaline cartilage, gingiva
VI	$\alpha1(VI)\ \alpha2(VI)\ \alpha3(VI)$	Descemet's membrane, skin, nucleus pulposus, heart muscle
VII	$(\alpha1(VII))_3$	Skin, placenta, lung, cartilage, cornea
VIII	$\alpha1(VIII)\ \alpha2(VIII)$ chain organization of helix unknown	Produced by endothelial cells, Descemet's membrane
IX	$\alpha1(IX)\ \alpha2(IX)\ \alpha3(IX)$	Cartilage
X	$(\alpha1(X))_3$	Hypetrophic and mineralizing cartilage
XI	$1\ \alpha2\ \alpha3\ \alpha1$ or $\alpha1(XI)\ \alpha2(XI)\ \alpha3(XI)$	Cartilage, intervertebral disc, vitreous humour
XII	$(\alpha1(XII))_3$	Chicken embryo tendon, bovine bovine periodontal ligament
XIII	Unknown	Cetal skin, bone, intestinal mucosa

Collagen is found in the extracellular matrix (ECM); it is one of the principal ingredients of the ECM. The ECM is defined as all the material of the tissue except the cells. In a dense connective tissue, such as tendon, ligament, or bone, the majority of tissue consists of ECM. The ECM consists of a network or mesh of interfibrillar matrix material containing proteoglycans and glycosaminoglycans.

Extracellular matrices are synthesized from the cells situated within them, for example, fibroblasts and osteoblasts, and numerous epithelial cells make particular types of collagen for their respective tissues. In the manufacture of the polypeptide chain, any combination of 20 different common amino acids may be incorporated, according to the genetic instructions of the cell. The amino acid residues may be modified subsequently so that the chains may contain a much wider variety of residues, amounting to nearly 200. The primary structure of the peptide is the order of the specific amino acid residues; for example, part of a collagen sequence is Gly-Ala-Hyp-Gly-Pro-Hyp-Gly-Ala-Hyp-Gly-Ala-Hyp-Gly-Pro-Val-Gly-Pro-Ala-Gly-Lys-Ser-Gly-As p-Arg-Gly-Glu-Thr-Gly-Pro-Ala-Gly, where the standard three-letter abbreviations for the amino acids have been employed. A collagen may be recognized by its amino acid composition, i.e., the relative frequencies of the different amino acid residues. The formation of collagen follows the general manufacturing process of proteins before being specialized as collagen. The manufacture of proteins begins with the process of “transcription” in the nucleus of a cell (Fig. 1).

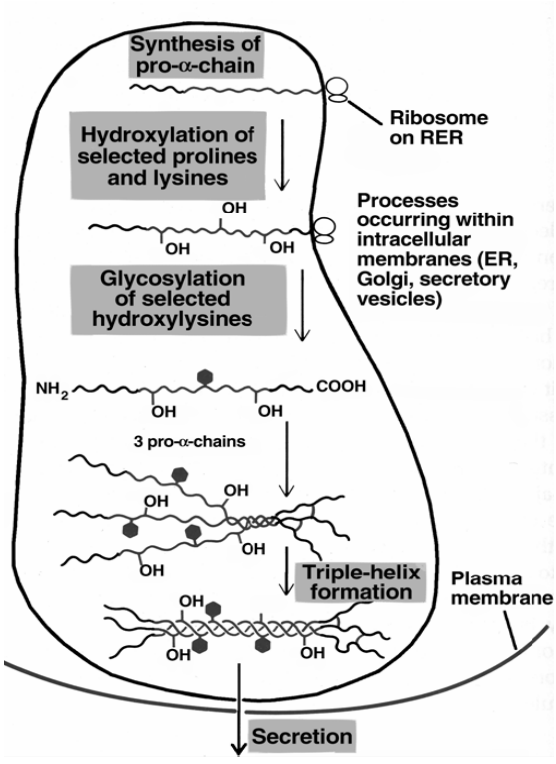


Fig. 1 *Synthesis of collagen molecule in the cell [3].*

In this process a long mRNA (m for messenger) segment is formed in a pattern determined by the nucleotide sequence found in a DNA segment. The mRNA contains the information required to sequence the amino acids in a protein. The mRNA passes out of the nucleus and

into the cytoplasm of the cell. Protein synthesis requires transfer RNA (tRNA) and ribosomal RNA (rRNA) in addition to mRNA, and it occurs at a ribosome on the rough endoplasmic reticulum. The process of "translation" is the synthesis of polypeptide α-chains at the ribosome using the information contained in the mRNA molecules. The process of hydroxylation and glycosylation then occur, followed by the rough assembly of the α-chain. Glycosylation is the substitution of one or more glycosyl groups. The flattened sacks of the endoplasmic reticulum are called the cisternae; it is here that the α-chains are released from the ribosome and form the triple helix of collagen. The helical structure travels from the cisterna of the endoplasmic reticulum to the Golgi apparatus, where the procollagen is packaged and condensed into a dense membrane-bound granule. The cytoskeletal system moves these granules to the cell membrane, where there is fusion between the external membrane of the cell [3].

2.1.1 Biomedical Applications of Collagen

Collagen is an important biomaterial in medical applications due to its special characteristics, such as biodegradability and weak antigenecity [4].

The use of collagen as a biomaterial is currently undergoing a renaissance in the tissue engineering field. The biotechnological applications focus on the aspects of cellular growth or delivery of proteins capable of stimulating cellular response. However, basic knowledge about collagen biochemistry and the processing technology in combination with understanding of the physico-chemical properties is necessary for an adequate application of collagen for carrier systems. The most successful and stimulating applications are shields in ophthalmology, injectable dispersions for local tumor treatment, sponges carrying antibiotics and minipellets loaded with protein drugs [2, 5].

The primary reason for the usefulness of collagen in biomedical application is that collagen can form fibers with extra strength and stability through its self-aggregation and cross-linking. In most of drug delivery systems made of collagen, in vivo absorption of collagen is controlled by the use of crosslinking agents, such as glutaraldehyde, chromium tanning, formaldehyde, polyepoxy compounds, acyl azide, carbodiimides, and hexamethylenediisocyanate [2, 5].

2.1.1.1 Advantages and disadvantages of collagen as a biomaterial

Advantages

- Available in abundance and easily purified from Libiny organisms (constitutes more than 30 % of vertebrate tissues)
- Non-antigenic
- Biodegradable and bioreabsorbable
- Non-toxic and biocompatible
- Synergic with bioactive components
- Biological plastic due to high tensile strength and minimal expressibility
- Hemostatic - promotes blood coagulation
- Formulated in a number of different forms
- Biodegradability can be regulated by cross-linking
- Easily modifiable to produce materials as desired by utilizing its functional groups
- Compatible with synthetic polymers

Disadvantages

- High cost of pure type I collagen
- Variability of isolated collagen (e.g. crosslink density, fiber size, trace impurities, etc.)
- Hydrophilicity which leads to swelling and more rapid release
- Variability in enzymatic degradation rate as compared with hydrolytic degradation
- Complex handling properties
- Side effects, such as bovine spongeform encephalopathy (BSF) and mineralization

2.2 Structure of collagen

The structure of collagen is described as a "coiled-coil" because it is a coil that is itself composed of coils. Thus, the result is the right-handed superhelix that is a coiled coil with a pitch of approximately 30 amino acid residues or 8.55 nm (Tab. 2). The reversal of twist at the next higher structural level is a technique used in the manufacture of rope and cable - it makes for a tighter structure that produces greater tensile strength [3].

Tab. 2 *Structural parameters of the collagen triple helix [3].*

	Collagen triple helix
Sequence repair	$(Gly\text{-}X\text{-}Y)_n$
Helix parameters	
Handedness	Left-handed
Residues per helix turn, *n*	3.33
Rise/residue, *d* (nm)	0.286
Helix radius, (nm)	0.15
Superhelix parameters	
Handedness	Right-handed
Supercoil radius, R_0 (nm)	0.28
Supercoil pitch (nm)	8.55
Mean mass-per-length ratio $(nm)^{-1}$	1000

Collagen molecules consist of three polypeptide chains, each coiled in a left-handed helix. The three chains are thrown into a right-handed triple superhelix stabilized by periodic hydrogen bonds. The triple helices, known also as tropocollagen, associate laterally and longitudinally to form microfibrils. These, in turn, form fibrils - aggregates of which are constituted various forms of connective tissue. Decrease of pH below 4 results in dissolving of the fibrils and formation of a molecular solution of tropocollagen [1].

2.2.1 Primary and secondary structure

Collagen is a protein. Proteins are linear polymers of amino acid residues linked by peptide bonds in a specific sequence. An amino acid is any organic acid containing one or more amino substituents. Amino acid residues are the amino acids that have been removed during the formation of peptide bonds. A peptide is any compound containing two or more amino acid

residues joined by a peptide bond, that is to say, a bond formed between the amino group ($-NH_2$) of one amino acid and the carboxyl group (-COOH) of another. The primary structure of peptides and proteins refers to the linear number and order of the amino acids present [3].

The basic collagen molecule contains three polypeptide chains, each consisting of more than 1000 amino acids [2]. The composition of the *α*1(I) and *α*2(I) chains of calf-skin collagen are given in Tab. 3 [2].

Tab. 3 *Amino acid composition of type I collagen from calf-skin (the values in parentheses are the residues contributed by the non-helical telopeptide regions) [2].*

Amino acid	***α*1(I)-chain**	***α*2(I)-chain**
Alanine	124 (2)	111 (3)
Arginine	53 (2)	56 (1)
Asparagine	13	23
Aspartic	33 (3)	24 (2)
Glutamic	52 (2)	46 (2)
Glutamine	27 (3)	24 (1)
Glycine	345 (6)	346 (6)
Histidine	3 (1)	8
Hydroxylysine	4	9
Hydroxyproline	114	99
Isoleucine	9 (1)	18
Leucine	22 (3)	33
Lysine	34 (2)	21 (1)
Methionine	7	4
Phenylalanine	13 (1)	15 (3)
Proline	127 (4)	108 (1)
Serine	37 (5)	35 (1)
Threonine	17 (1)	20
Tyrosine	5 (5)	4 (3)
Valine	17 (1)	34
Total	**1056 (42)**	**1038 (24)**

There are only minor differences between the collagen from different vertebrate species. Glycine (Gly) has the smallest side group and repeats at every third position on the sequence. About 35 % of the non-glycine positions in the repeating unit Gly-X-Y are occupied by proline (Pro), found almost exclusively in the X-position, and 4-hydroxyproline (Hyp), predominantly in the Y-position (Fig. 2a) [2].

The *α*-chains combination forms left-handed helices with 3.3 residues per turn and a pitch of 0.87 nm as identified by X-ray analysis (Fig. 2b) [2].

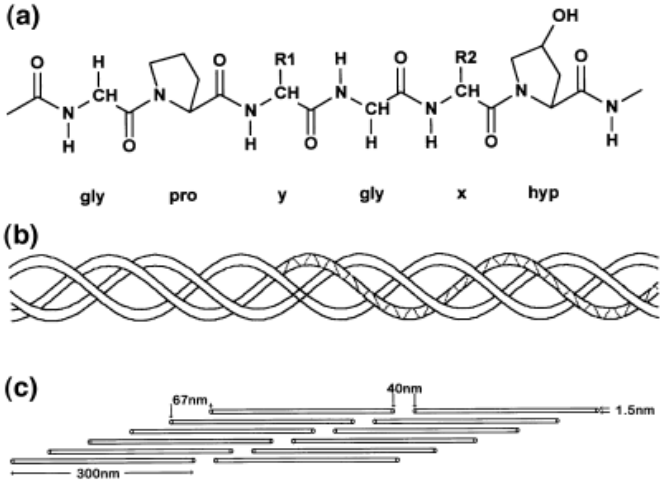


Fig. 2 *Chemical structure of collagen type I. (a) Primary amino acid sequence, (b) secondary left handed helix and tertiary right handed triple-helix structure and (c) staggered quaternary structure [2].*

2.2.2 Tertiary and ternary structure

The tertiary structure of collagen consists of three (polypeptide) *α*-chains of about 1000 amino acid residues at each. It is a right-handed superhelix composed of the three *α*-chains (Fig. 2). Two of the *α*-chains are identical, but the third one is slightly different with respect to its amino acid sequence (i.e., two alpha 1 and one alpha 2 chains). The tertiary structure refers to the fundamental unit originally known as tropocollagen about pitch of approximately 8.6 nm. The rod-shaped triplehelix has an average molecular weight of approximately 300 kDa, a length of 300 nm with a diameter of 1.5 nm (Fig. 2c) [2].

This extreme ratio of the dimensions gives rise to high viscosity in solutions and high mobility in electrical fields. In addition, there are regions of 9 - 26 amino acids at the amino and carboxyl terminal chain ends of the molecule that are not incorporated into the helical structure. These non-helical regions are denoted as telopeptides [2].

A requirement for the formation of the superhelix is the repeating sequence -Gly-Xaa-Yaa-. A straight not supercoiled polyproline-II-helix has exactly three residues per turn and glycine residues are facing each other in the interior of the triple helix (Fig. 3B) [6].

Larger side chains at the C*α*-atoms than H would present formation of a hydrogen bond between the backbone N-H group of glycine and the backbone C=O of a residue in X-position of a neighboring chain. Isolated polyproline-II-helices are not stable if the polypeptide chains also contain other residues than proline and hydroxyproline [6].

The prefix super is used because each of the three *α*-chains is itself wound into a left-handed helix with three amino acids in each turn of the helix. Thus, right-handed super-helix means a helix of a structure that is itself a left-handed helix [3].

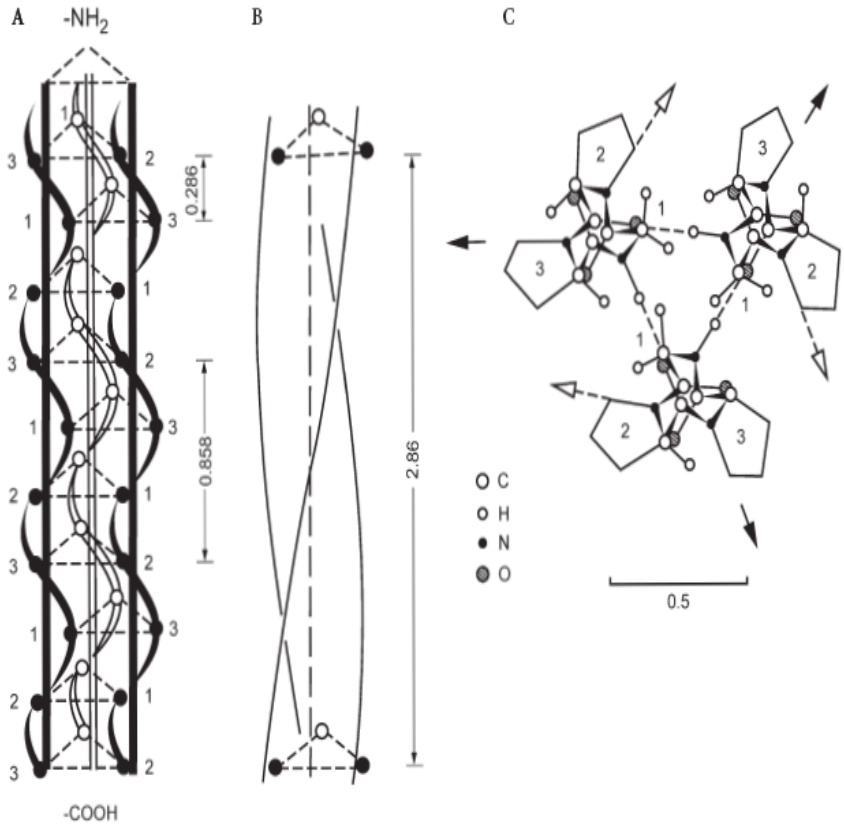


Fig. 3 *Model of the collagen triple helix. The structure is shown for (Gly-Pro-Pro)$_n$ in which glycine is designated by 1, proline in X-position by 2 and proline in Y-position by 3: A, B side views. Three left-handed polyproline-II-type helices are arranged in parallel. For clarity the right-handed supercoil of the triple helix is not shown in A but indicated in B. Dashed lines indicate positions of Cα-atoms (and not hydrogen bonds as in C). All indicated values for axial repeats correspond to the supercoiled situation; C top view in the direction of the helix axis. The three chains are connected by hydrogen bonds between the backbone N-H of glycine and the backbone C-O of proline in Y-position (dashed lines).Arrows indicate he directions in which other side chains than proline rings emerge from the helix. Approximate residue-to-residue distances, repeats of the polyproline-II- and triple helix and a scale bar are indicated in nm [6].*

On the fourth level of order, the triple-helical molecules stagger longitudinally and bilaterally into fibrils with distinct periodicity. The collagen molecules aggregate through fibrillogenesis into microfibrils consisting of four to eight collagen molecules and further into fibrils. Those fibrils reach from 10 to 500 nm in diameter depending on tissue type and stage of development. The triple-helices are staggered by 67 nm with an additional gap of 40 nm between succeeding molecules. These collagen fibrils organize into fibers, which on their part can form even larger fiber bundles [2].

Type I collagen is presented in the extracellular matrix of connective tissues in the form of fibrils and fibres that have a characteristic arrangement of the single triple helical molecules. On negative staining, fibrils show a regular pattern of dark (gap zone) and light bands (overlap zone) and on positive staining a regular pattern of sharp dark bands related to the presence of charged residues, i.e. to the amino acid sequence. The overlap zone has a higher content of

basic residues than of acidic ones, whereas there is no predominance of negative or positive residues in the gap zone. It follows that the overlap zone is more sensitive to pH variations than the gap zone, and the greater stability of the former at neutral pH with respect to acidic pH is due to entropic factors [7].

2.2.3 Atelocollagen

Atelocollagen is a telopeptides-free collagen molecule. It is true that the enzymatic procedure used to remove the telopeptides did not change the ability of the collagen to aggregate structures and keep substantially unchanged natural properties of the original molecules as the stability in contact with biological fluids. This is good information to strengthen the safety in the commercial use of these products. This observation and the unchanged free-amino groups suggest that the telopeptides free collagen self-assembled in fibres and the interaction were stabilized by the spontaneous new formation of intermolecular cross-links but the sponges prepared by freeze dried process showed a different water absorption trends in time and capacity with respect to those formed by intact collagen. This behaviour could be ascribed at a different organization of the collagen fibres to form the three-dimensional structure of the sponge; the sponges showed an alveolar structure in which pores defined the real capacity to interact with cells and also biological fluids. The irregularity and the major dimension of pores give the atelocollagen sponge more water sensitivity because porosity is directly and quickly accessible to the water that was easily absorbed and kept in the structure. The evaluation of morphology and the physico-chemical characteristics of atelocollagen sponges were very important to evaluate the potential capacity in cell-cell and cell-sponge interactions [8].

2.3 Physical properties of collagen

Molecular weight distribution of collagen is very narrow due to the average molecular weight of about 200 and 300 kDa for β and α-chains, respectively, compared with wide distribution of gelatin having average molecular weight less than 95 kDa.

Gelatin is the denatured product of native collagen and adopted a random coil conformation, showing different properties, e.g. relative lower molecular weights and wide molecular distributions, lower isoelectric points without conformation translation, loss of ability of fibril formation, and being easily attacked by proteinase. Gelatin can be made into roll film and drug capsules and can also be used as a biomaterial in biomedical applications, such as in drug delivery systems which are very different from the traditional capsule [4].

The isoelectric points of collagen and gelatin were 8.26 and 4.88, respectively, determined by Zeta potential titration. Circular dichroism (CD) spectra revealed that there were two peaks, a positive peak around 221 nm and a negative peak around 192 nm for collagen, which are the characteristics of collagen triplehelix. However, gelatin lacked any positive peaks around 220 nm, suggesting random coils. The denaturation temperature of collagen was about 37.5 °C determined by the viscosity method, the helix-coil transitions for gelatin were not presented in the heating process. Collagen reaggregated into fibrils at 35 °C monitored at 313 nm. In contrast, gelatin lost the ability of fibril formation. Collagen was more resistant to trypsin hydrolysis compared with gelatin [4].

2.3.1 Stability of collagen structure

The intra- and interchain bonding or crosslinking between specific groups of the chains is essential to the stability of the molecule. Glycine enhances the stability of the molecule by forming hydrogen bonds among the three chains of the superhelix. Hydroxyproline and proline form hydrogen bonds or hydrogen-bonded water bridges within each chain. Lysine and hydroxylysine stabilize the triple helix. Due to their alicyclic nature they stiffen the *α*-chain and form hydrogen-bonds limiting rotation [2, 3].

In addition, there exist covalent intramolecular bonds, which link two or three peptide *α*-chains of the same molecule and give rise to the appearance of *β*- (2 *α*-chains) and *γ*- (3 *α*-chains) components in chromatographic and electrophoretic patterns. The intramolecular linkages exert no influence on the stability of the fibrils. Bornstein et. al. [9] was able to demonstrate that two lysine residues are involved in an intramolecular bond. These residues are located in the non-helical N-terminal region of the *α*-chains. The lysine residues are first oxidized to *α*-aminoadipine-*δ*-semialdehyde. The cross-linkage is brought about by aldole condensation between aldehydes on two different chains and subsequent dehydration.

Tanzer and Bailey [10, 11] presented evidence for the presence of intermolecular bonds of the Schiff's base type. Such bonds are sensitive to acids, but they can be stabilized by reduction with sodium borohydride. Bailey et. al. [12] isolated $\Delta^{6,7}$-dehydrohydroxylysinonorleucine from reduced collagen. They deduced, therefore, that the corresponding intermolecular bond had been of the aldimine type and had originated from the reaction of the aldehyde group of a lysinealdehyde with the amino group of a hydroxylysine. According to Bailey et. al [12]. an additional bond, which is acid stable, is formed by an aldol condensation of the *δ*-semialdehyde of the lysine and hydroxylysine [9].

Trans-4-Hydroxy-*L*-proline (Hyp) makes a specific and significant contribution to stability of the triple helix, whereas the *cis* isomer decreases the stability. What is still controversial is if bound water contributes to the stability of collagen triple helix. Hyp has attributed a great relevance to because this cyclic amino acid can serve as a coordination centre of an extensive network of water molecules [7].

Raines and coworkers [7] pointed the inductive effects of the Hyp hydroxyl group as the source of stability. This conclusion was based on structural, thermodynamic and kinetic aspects, summarised in the simplest way by the rise of the melting temperature of the trimeric species. This species are formed by the synthetic collagen-like peptide $(Pro\text{-}Hyp\text{-}Gly)_{10}$ when Hyp OH groups were substituted by highly electronegative fluorine atoms. Berman and coworkers [7] reported crystallographic data on a 30-mer synthetic peptide that incorporates an imino acid-poor natural collagen sequence. These authors confirmed the set of direct interchain H bonds between N-H (Gly) and O=C (X position). In the imino acid-poor region, they also obtained evidence of a second set of repetitive backbone interchain hydrogen bonds between amide N-H (X position) and O=C (Gly). Each bond was found to be mediated by a single water molecule integral to the triple helical structure and was judged almost as strong as the former direct interchain H bonds. The characteristics of the second set of H bonds were considered consistent with earlier solution studies on hydrogen exchange. This implies that water molecules have a structural role in triplehelix both in the solid state and in solution. Residence time of water strongly bound to collagen is about 1 μs, i.e. still in a rapid exchange with the bulk of water.

Almost all determinations of the stability of fibrillar collagens, mostly on type I collagen, were performed at acidic pH because these proteins are soluble in this condition. Fibrils can not be formed because at acidic pH the force of the interaction between triple helices has no attractive

component at any inter-helical distance. On the contrary, at physiological conditions of pH, ionic strength and composition of the collagen solution, the triple helical melting at increasing temperatures, is accompanied by the formation of fibrils and then by their disruption [7].

Type I collagen is thermodynamically rather kinetically stable, but only below body temperature. The equilibrium T_m of collagen monomers in physiological solution is several degrees below body temperature (T_m < 36 °C for human lung and 28 °C < T_m < 35 °C for rat-tail-tendon collagen). The melting time at body temperature is from several hours (rats) to several days (humans) [14].

Upon heating, collagen undergoes a denaturational transition from the triple helix to a randomly coiled form in 40.88 °C. In this transition the three chains are separated and the transition is not reversible upon immediate reheating [15]. A correlation between chemical composition and thermal stability of collagens has been established [1].

2.3.2 Collagen and water

Collagen is a highly hydrated protein. For preservation of physical properties of collagen it is necessary to associate a minimal amount of water. This amount of water forms approximately 20 % of collagen total weight [16]. In a crystal formed by a collagen-like peptide, adjacent triple helices have little or no direct contact; the connections are maintained by hydrogen-bonded water bridges. Water-mediated hydrogen bonding between polar residues was indicated as the cause of type I collagen self-assembly [7].

In fully hydrated state, collagen releases a movement constriction of peptides chains, which is typical for dry state. It explains an elementary function of water for physical properties of collagen. Distance between two neighbouring polypeptides chains is 1 nm for dry collagen and 1.5 – 1.6 nm for hydrated collagen [16].

From physico-chemical aspect collagen is counted among transitive colloid systems - gels. The most important property of gels is their ability to absorb water. If the collagen fibre is dunked to water, a limited extent swelling (exoterm process) has began. One part of water presented in swelling collagen is mechanically removable and the second part - the hydrating water - colloidly bounded, which can be removed by drying only. During the swelling process the fibre transforms its volume, length and flexibility [16].

Swelling ability of collagen is possible to measure by swelling ratio (SR), which is formulated by equation (1), where W_s is weight of swelling material and W_d is weight of dry material.

$$\mathrm{SR} = \frac{\mathrm{W_s}}{\mathrm{W_d}} \tag{1}$$

The ability of a collagen sponge to preserve water is an important aspect to evaluate its property for tissue engineering. Relevant differences are detected between the water binding capacity of equine intact collagen and telopeptides-free collagen sponges (atelocollagen). Atelocollagen sponges showed a significant increase in water absorption compared with the intact collagen samples. Dependence of the water absorption capacity, expressed as ml of water absorbed per cm^2 of sponges, on the time is shown in Fig. 4. The trends of the curves confirm the different behaviour with respect to the water absorption capacity between the intact and atelocollagen sponges. All samples rapidly absorbed great amount of water, but only in 60 min

the intact collagen sponges were quite imbued with water. The atelocollagen sponges resulted in the water maximum absorption in 6 – 7 h [8].

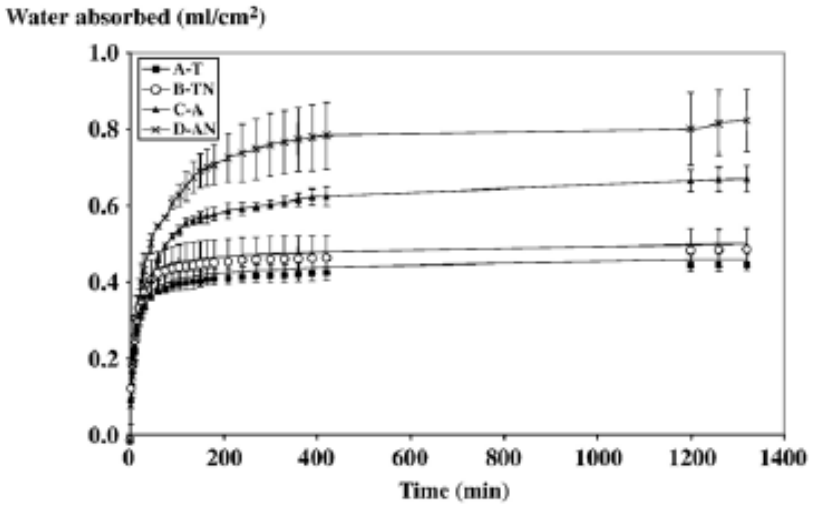


Fig. 4 *Water absorption dependence on the time. Type I equine intact (A-T and B-TN) and telopeptides-free collagen (C-A and D-AN) [8].*

2.3.2.1 Specific volume changes in calf skin collagen type I

During the heating of collagen fibres in water, the fibers become shorter by one third in direction of its longitudinal axis approximately. The termic contraction is characterized by temperature of contraction (T_s). Causation of contraction is splitting of transverse intermolecular bonds as well as intramolecular bonds, which keep the triple helix in stretched state. If the intramolecular bonds are disrupted, collagen begins denaturation. Contraction of collagne fibers is endotermic process that can be explored by thermal analytical methods (DSC). T_s of collagen is considered on behalf of melting of collagen in crystallin areas. Contraction of collagen fibres and decreasing of T_s are also caused by lyotrophic reagents, which split the hydrogen bonds [16].

The splitting of the collagen denaturational transition into two components upon oxidation become evident from the associated specific volume changes (Fig. 5). The transition is not reversible upon cooling and immediate reheating, in accordance with the calorimetric data. Within heating of an oxidized sample, two steps in the specific volume were observed at approximately 35 and 40 °C (Fig. 5b), in good agreement with the respective specific heat curves. The total specific volume increase was approximately between 0.02 – 0.03 ml.g^{-1}. The stepwise specific volume increase accompanying the denaturation of collagen may result in the protein aggregation, disruption of the ion pairs between neighbouring acidic and basic amino acids (formation of such pairs in the native state is known to reduce their volume due to electrostriction), or in combination of these two effects. As it is clear from Fig. 5, the stepwise increases of the specific volume upon denaturation are not reversible in cooling direction. Interestingly, the specific volume of collagen markedly increases upon denaturation, by contrast with the thermal denaturation of globular proteins, which is known to take place with very small or no specific volume changes [15].

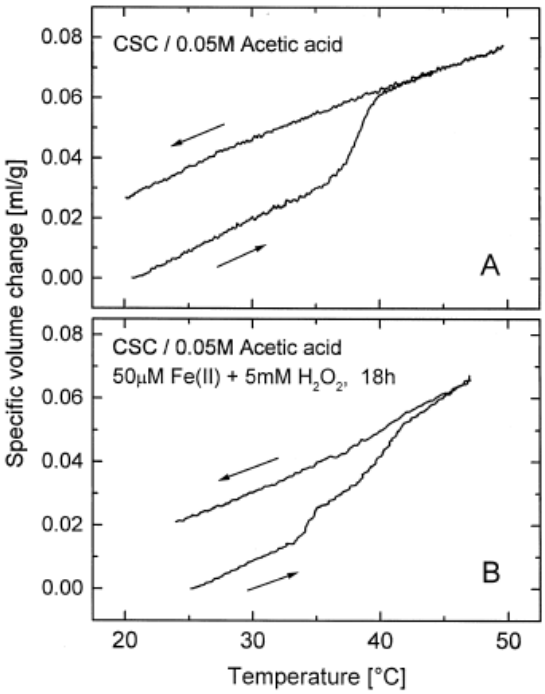


Fig. 5 *Densitometric heating and cooling traces of calf skin collagen type I in 0.05 M acetic acid, recorded at 0.5 °C min. (a) fresh, untreated CSC preparation, (b) CSC preparation after 18 h incubation with 50 μM Fe(II) 5 mM H_2O_2 at 22 °C [15].*

2.3.3 Solubility of collagen

The most common types of collagens occur in fibres and networks.These proteins are poorly soluble (if at all), are found in many tissues such as connective tissue and have a slow metabolic turnover. This is the reason why they are more susceptible to some enzymatic or nonenzymatic posttranslational modifications. Polymerized fibril-forming collagens (whether polymerized physiologically or non-physiologically) are insoluble and their solubilization is routinely performed either by mild pepsinization, in which short terminal regions possessing the polymerization sites (cross-links) are cleaved off, or by CNBr cleavage, which results in a limited fragmentation of the parent *α*-chains as mentioned already. Tissue collagenases (which unfortunately are difficult to obtain) split the triple-helical structure two-thirds of the way from its N-terminus; bacterial collagenases (from *Clostridium histolyticum)* are far less specific, they cleave the sequence in small fragments (mostly tripeptides) and are therefore of little use in structural studies [17].

Lack solubility of collagens is due to covalent bonds which interconnect the molecules within the collagen fibrils. These intermolecular bonds develop in a process of slow maturation following aggregation of the molecules [7]. The major impediment to dissolution of collagen type I because of thus the presence of covalent crosslinks between molecules. Collagen is insoluble in organic solvents. Watersoluble collagen represents only a small fraction of total collagen and the amount depends on the age of the animal and type of tissue extracted. In some tissues, notably skin from young animals, crosslinking is sufficiently low to extract a few percent under appropriate conditions. Furthermore, collagen molecules present within fibrillar aggregates can be dissociated and brought into aqueous solution. However, the nature of the crosslinks

prevalent in different tissues determines the particular solvent to be used and the corresponding yields [2].

Using suitable concentration the collagen exist soluble in diluted solutions of organic acids in the form of thin elongate stisks, which lenght is 280 nm and diameter 1.6 nm. Their relative molecular weight range from 265 to 300 kDa. It describes a category of soluble collagens, which are obtained by extraction in cool slightly alcalic solutions of salts or neutral solutions of salts with hypertonic and physiologic force. In native form, only aggregation forms of collagen having no intramolecular covalent bonds, is possible to dissolve. Insoluble collagen from aged tissues can be dissolved after previous modification. During modification part intermolecular transverse bonds are split and fractional or complete chemical or thermal denaturation begins. From tissues of young individual 10 – 15 % of collagen can be dissolved and with ageing of connective tissue the solubillity decreases [6]. Recent studies indicated that for use the collagen as biomaterial alcalic modification is more effective than acidic one because alcalic modification positively influences the thermal stability of collagen and rule out form of fibrils in neutral pH and in physiological conditions [16].

2.3.3.1 Neutral salt soluble collagen

The most commonly used solvents are neutral salt solution (0.15 – 2.0 M NaCl) or diluted acetic acid. Neutral salt solutions will extract freshly synthesized and negligibly crosslinked collagen molecules presented in tissue. Modifications in temperature, shaking rate, and volume of extractant to tissue ratio will inevitably derived alter the composition of the collagen. The extracted material is purified by dialysis, precipitation, and centrifugation. Most tissues have little or no salt-extractable collagen. In order to increase the yield for research purposes animals can be fed with *β*-aminopropionitrile - the inhibitor of peptidyl lysyl oxidase - however, this procedure is inadequate for larger commercial scale [2].

2.3.3.2 Acid soluble collagen

Dilute acidic solvents, e.g. 0.5 M acetic acid, citrate buffer, or hydrochloric acid of pH 2 - 3 are more efficient than neutral salt solutions. The intermolecular crosslinks of the aldimine type are dissociated by the dilute acids and the repulsive repelling charges on the triple-helices lead to swelling of fibrillar structures. Dilute acids will not disassociate less labile crosslinks such as ket-imine bonds. Therefore collagen from tissues containing higher percentages of keto-imine bonds, i.e. bone, cartilage, or material from older animals have a lower solubility in dilute acid solvents. In order to acid extract collagen, generally, tissue is grounded in the cold, washed with neutral saline to remove soluble proteins and polysaccharides, and the collagen extracted with a low ionic strength, acidic solution. It is possible to solubilize approximately 2 % of the tissue collagen with dilute salt or acid solutions. These collagen molecules can be reconstituted into large fibrils with similar properties as native fibrils by adjusting the pH or temperature of the solution. The remaining 98 % is referred to as insoluble collagen although this dominant collagen material is not absolutely insoluble and can be further disintegrated without major damage to the triple-helical structures. The two most common approaches are the use of strong alkali or enzymes to cleave additional crosslinks and suspend or dissolve acid-insoluble structures [2].

2.3.3.3 Alkali- and enzyme-treated collagen

Collagen material can be solubilized by treating connective tissue with an aqueous solution comprised of alkali hydroxide and alkali sulfate, e.g. approximately 10 % of sodium hydroxide and 10 % of sodium sulfate for approximately 48 hours. Thus, fat associated with the insoluble collagen is saponified, non-helical telopeptide regions are truncated and the collagen fibers disintegrated. The size and molecular weight of the resulting collagen material depend on the time of treatment and alkali concentration. The presence of alkali sulfate controls the swelling of the collagen structures and protects the native triple-helical characteristics. It has to be noted that similar to gelatin, the isoelectric point of the resulting material is shifted to lower pH as asparagine and glutamine are converted into aspartic and glutamic acid. Much higher yields compared with acidic extraction can be achieved by taking advantage of the fact that the collagen triple-helix is relatively resistant to proteases, i.e. pronase, ficin, pepsin or chymotrypsin below approximately 20 °C. The efficacy of enzymatic treatment arises from selective cleavage in the terminal non-helical regions breaking peptide bonds near crosslinks and releasing molecules which dissolve. Some crosslinks presumably remain, attaching small peptide remnants to the solubilized molecules. Thus, the telopeptide ends of the polymer chains are dissected but under appropriate conditions the helices remain essentially intact. The resulting material, so-called atelocollagen, benefits from the removal of the antigenic P-determinant located on the non-helical protein sections and provokes milder immune response. Pepsin at a 1:10 weight ratio of enzyme to dry weight tissue in dilute organic acid (0.5 M acetic acid) provides an propitious medium in which collagen can be swollen and solubilized. Soluble collagen is purified mainly by precipitation after pH, salt concentration or temperature adjustment. The high viscosity of even dilute solutions interferes with purification methods such as chromatography, electrophoresis and differential sedimentation. Collagen solutions contain varying proportions of monomer and higher molecular weight covalently linked aggregates, depending on the source and method of preparation. Truly monomeric solutions are difficult if not impossible to obtain. Pepsin-solubilized collagen usually contains higher proportions of monomer than salt or acid extracted material. Soluble collagen can be stored frozen or lyophilized. In the course of drying, denaturation or non-specific crosslinking can occur and the degree of association upon reconstitution can change [2].

2.3.3.4 Insoluble collagen

Instead of disintegration and transfer into soluble material, extensively crosslinked collagen can be dispersed as opalescent, fine fibrillar suspensions by the use of mild denaturation agents and mechanical fragmentation usually at an acidic pH. Fibrillar collagen is more resistant to proteolysis than most other non-collageneous tissue constituents, which are removed during processing by selective proteolysis and washing. In additional steps collagen material can be subjected to chemical modifications such as succinylation, acetylation, methylation or attachment to other polymers [2].

2.3.4 Mechanical properties of collagen

There have been at least six efforts to experimentally estimate or determine the axial Young's modulus of a collagen molecule over the last thirty years. These studies have used collagen from different collagen-containing tissues and they have employed different experimental techniques. The measurements of Young's modulus have produced values from

about 3 to 9 GPa (Tab. 4). This range of moduli at the low end corresponds to the Young's modulus of Teflon and at the upper end, the modulus of wood, fairly rigid for an organic molecule [3, 18].

Tab. 4 *Measurements of Young's modulus for healthy and pathological collagen molecules from various tissues by various methods [3].*

Tissue form which the collagen was obtained	**State**	**Nature of the test procedure**	**Young's modulus (GPa)**	**Source**
Bovine Achilles tendon	Solid state in 0.15 M NaCl solution	Static X-ray diffraction	2.9 ±0.1	Sasaki & Odijama (1996) [19]
Rat tail tendon	Solid state in 0.15 M NaCl solution	Brillouin scattering	9.0	Harley et. al. (1977) [20]
Rat tail tendon NaCl solution	Solid state in 0.15 M NaCl solution	Brillouin scattering	5.1	Cusack & Miller (1979) [21]
Lathyritic rat skin	Acetate/NaCl/glycerol solution	Dynamic measurement of persistence lenght	4.1	Nestler et. al. (1983) [22]
Dermatosparaxie calf skin	0.05 M acetic acid glycerol solution	Static measuremets of persistence lenght	5.1 – 3.0	Hofmann et al. (1984) [23]

It is possible to attribute the differences in the values of Young's moduli in Tab. 4 to several factors. First, there is the difference in the properties of the collagen from different tissues, some of which were not "normal" tissues. Second, there is the difference between the "static" and "dynamic" experimental methods that might be related to the viscoelasticity of the tissue and to the breaking and forming of bonds. In spite of these considerations, measurements of Young's modulus of collagen have produced values that are not too greatly varied given the difficulty of the problem.

At strains typically beyond 3 %, the stiffness of rat tail tendon increasing considerably with the extension (Fig. 6). The intensity of the diffuse equatorial scattering, which is due to the lateral arrangement of the collagen molecules inside the fibrils increased linearly with the strain. This was interpreted as a reduction of the disorder in the lateral molecular packing within fibrils, resulting from the straightening of kinks in the collagen molecule [18].

Indeed, kinks are though to occur within the gap region of the collagen fibril structure. In particular, a recent refinement of the collagen fibril packing structure point toward the existence of kinks. They might occur in the gap region of the collagen fibril because of the greater flexibility of collagen molecule due, first, to lower levels of proline and hydroxyproline on the collagen chain and, second, to the reduced packing density as compared to the overlap region. Moreover, considerable azimuthal and lateral flexibility of collagen molecules hed been demonstrated in NMR measurements [18].

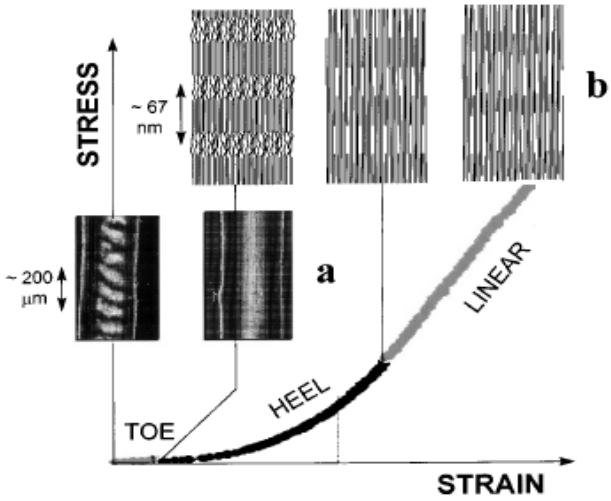


Fig. 6 *Typical stress-strain curve of a rat tail tendon. In the toe region, where the tendon can be extended with very little force, a macroscopic crimp of the fibrils with a typical period in the order of 100 µm is removed. This can be visualized using polarized light (a). Further structural changes occur at the fibrillal level (b). The heel region may correspond to a straightening molecular kinks in the gap and the linear region to a gliding of molecules. The most recent synchrotron diffraction data suggest that a disruption of the fibrilar structure starts with an increased fuzzines of the gap/overlap interface [18].*

When collagen is stretched beyond the heel region of the stress/strain curve, most kinks are straightened and no further extension is possible by the entropic mechanism described above. Therefore, some other process must prevail in the linear region of the stress/strain curve. The most likely processes are a stretching of the collagen triple-helices or of the cross-links between the helices, implying a side by side gliding of neighboring molecules. This process has already been studied in the mid-eighties by use of synchrotron radiation diffraction experiments. In these experiments, a strain-induced change in the structure factor of the axial diffraction maximums was observed. In particular, the second order maximum increased with respect to the third order, when the tendon was stretched. This was a clear indication that stretching increased the lenght of the gap region with respect to the lenght of the overlap region, implying a considerable gliding of neighboring molecules [18].

2.4 Denaturation

In the denaturation process the triple helix of native collagen can be transformed to the random coil configuration. Simultaneously the physical properties such as viscosity, solubility and optical activity changed due to the collapse of the triple helical structure [4].

Denaturation of proteins involves the disruption and possible destruction of the secondary, tertiary and ternary structures. Since denaturation reactions are not strong enough to break the peptide bonds, the primary structure (sequence of amino acids) remains the same after a denaturation process. Denaturation disrupts the normal alpha-helix and beta sheets in a protein and uncoils it into a random shape. Denaturation occurs because the bonding interactions

responsible for the secondary structure (hydrogen bonds to amides) and tertiary structure are disrupted.

In ternary collagen structure disruption of three-dimensional organization of collagen molecules in fibrile occurs. In tertiary structure of collagen there are hydrogen bonding interactions between "side chains" which may be disrupted. In secondary collagen structure hydrogen bonding occurs between amide groups [24].

By denaturation of collagen a gelatin springs up. Orechovič and Spikiter [25] discover that product of denaturation reaction of water solution of tropocollagen are two breaked aparts which have different molecular weight. These breaked aparts are components *α* and *β*. Component *β* can be next split in two components *α*. Thus during denaturation of water solution of tropocollagen three breaked aparts which corresponded to collagen triple helix are formed. Mechanism of tropocollagen denaturation has two steps. In first step the triple helix is destroyed and macromolecule of tropocollagen is contracted into statistical globule in which individual chains are still interconnected. In second step this globule is fragmented at three parts: part *α* is formed by one polypeptide chain of original spiral, part *β* is formed by two still interconnected chains and part *γ* is formed by three chains in unchanged statistical globule. If the denaturation solution of tropocollagen stays for a long time at low temperature the renaturation proces will start run [26].

2.4.1 Thermal denaturation

When proteins are exposed to increasing temperature, losses of solubility or enzymatic activity occurs over a fairly narrow range.

As the temperature is increased, a number of bonds in the protein molecule are weakened. Firstly, the long range interactions that are necessary for the presence of tertiary structure are affected. As these bonds are first weakened and are broken, the protein obtains a more flexible structure and the groups are exposed to solvent. If heating decreases at this stage the protein should be able to readily refold to the native structure. As heating continues, some of the cooperative hydrogen bonds that stabilize helical structure will begin to break. As these bonds are broken, water can interact with collagen and form new hydrogen bonds with the amide, nitrogen and carbonyl oxygens of the peptide bonds. The presence of water further weakens nearby hydrogen bonds by causing an increase in the effective dielectric constant near them. As the helical structure is broken, hydrophobic groups are exposed to the solvent.

The effect of exposure of new hydrogen bonding groups and hydrophobic groups increase the amount of water bounded by the protein molecules. Resulted unfolding increase the hydrodynamic radius of the molecule causing the increase in viscosity of the solution.

Upon cooling, the structures obtained by the aggregated proteins may not be those of lowest possible free energy, but kinetic barriers will prevent them from returning to the native format. Any attempt to obtain the native structure would first require broken of the hydrophobic bonds that caused the aggregation. This would be energetically unfavorable and highly unlikely. Only when all the intermolecular hydrophobic bonds were broken, could the protein begin to refold as directed by the energy of short range interactions. The exposure of this large number of hydrophobic groups to the solvent, however, presents a large energy barrier that makes such a refolding kinetically unlikely [24].

Denaturational transition of collagen type I occurs at 40.8 °C with calorimetric enthalpy *ΔH* of 49.7 $kJ.g^{-1}$ and with no trace of a pretransition at 35 °C. This transition is not reversible because collagen contains hydrogen bonds in secondary structure [14, 15].

2.4.1.1 Agents affecting collagen thermal denaturation

Presence of urea, glycerol or sodium chloride affects the thermal denaturation of collagen. The T_d (temperature of denaturation) values increase proportionally to the concentration of glycerol (Tab. **5**) in the reaction medium [27, 28]. The denaturation temperatures decreased monotonously with the increasing of urea concentration (about 3 °C per 1 M urea) [27]. Similarly as urea, sodium chloride decreased denaturation temperature of collagen molecule [28].

Tab. 5 *Denaturation temperatures of sheep skin collagen (SSC), rat tail collagen (RTC), human placenta collagen (HPC) and calf skin collagen (CSC) at different concentration of glycerol [27].*

Collagen	Glycerol [M]	Td [°C]	ΔHvH [kcal/M]
SSC	0	40.8	391.46
	1	41.4	374.24
	2	42.4	395.45
	3	43.0	396.96
	4	44.2	380.94
RTC	0	40.5	459.66
	1	41.2	436.06
	2	41.7	437.45
	3	42.7	417.06
HPC	0	40.7	355.64
	1	41.7	302.85
	2	42.6	316.76
	3	43.8	332.47
	4	44.4	333.74
CSC	0	40.7	326.00
	1	41.3	302.07
	2	42.6	304.65
	3	44.1	308.91

It was observed that in the presence of glycerol, the collagen molecule was stabilized not only toward heating, but also toward the action of chemical agents. Glycerol diminishes the denaturing effect of urea and sodium chloride [27, 28].

Glycerol inhibits the fibril formation of collagen type I [29], acting oppositely in comparison with the globular proteins. Na [30] suggests that the stabilizing effect of glycerol is achieved by its binding to the surface of the collagen molecule through incorporation of its hydroxyl groups into the water-chain structure. Possible targets of its binding might be hydroxyproline or polar amino acid residues [28]. In this way glycerol stabilizes the protein solvation shell and competes with water molecules, probably due to its greater ability for formation of hydrogen bonds; each molecule contains 3 hydroxyl groups. The presence of hydroxyl groups in the solvent structure is important for stabilization of collagen due to formation of additional, stabilizing hydrogen bonds. On the other hand, the large volume of the glycerol molecule makes impossible its incorporation

in the water-chain structure, as a replacement for water-mediated hydrogen bonds e.g. Hyp-OH-Gly-CO [30]. About 50 % of the Hyp-residues occupy the Yyy-position in the triplets. So they assumed that glycerol could form hydrogen bonds with Hyp-OH groups situated in two neighbouring triplets. This leads to stabilization of every individual polypeptide *α*-chain without increasing the number of interchain stabilizing hydrogen bonds [27, 28].

2.4.2 Denaturation by changes the pH

Most proteins at physiological pH are above their isoelectric points and have a net negative charge. When the pH is adjusted to the isoelectric point of the protein, its net charge will be zero. Charge repulsions of similar molecules will be at minimum and many proteins will precipitate. Even for proteins that remain in solution at their isoelectric points, this is usually the pH of minimum solubility.

If the pH is lowered far below the isoelectric point, the protein will lose its negative and contain only positive charges. The like charges will repel each other and prevent the protein from aggregating as readily. In areas of large charge density, the intramolecular repulsion may be great enough to cause unfolding of the protein. This will have an effect similar to that of mild heat treatment on the protein structure. In some cases the unfolding may be extensive enough to expose hydrophobic groups and cause irreversible aggregation. Until this occurs such unfolding will be largely reversible.

Some proteins contain acid labile groups and even relatively mild acid treatment may cause irreversible loss of function. This generally results from the breaking of specific covalent bonds and thus should be considered separately from denaturation. Exposure to strong enough acid at elevated temperatures will first release amide nitrogen from glutamine and asparagine groups and eventually lead to hydrolysis of peptide bonds.

The effects of high pH are analogous to those of low pH. The proteins obtain a large negative charge which can cause unfolding and even aggregation. The use of high pH to solubilize and alter protein structure is very important to the formation of fibers from proteins of plant origin.

A number of reactions can cause chemical modification of proteins at alkaline pH's that are commonly encountered in protein processing. Many of these involve cysteine residues. Perhaps the most important are the base catalyzed beta eliminations of sulfur to yield dehydroalanine which can react with lysine to form lysinoalanine. These results in a loss of nutritive value of the protein and the products of the reaction may be toxic. Exposure of protein molecules to high pH should be minimized as much as is possible. Exposure to very high pH at elevated temperatures results in alkaline hydrolysis of peptide bonds [24].

2.4.3 Changes in dieletric constant

The addition of a solvent that is miscible with water, but is less polar, will lower the dielectric constant of the system. This will tend to increase the strength of all electrostatic interactions between molecules that were in contact with water. Many of the protein hydrogen bonds are effectively removed from the solvent and will not be affected. The presence of the less polar solvent will also have the effect of weakening the hydrophobic bonds of the proteins. These bonds depend upon an increase in the order of water when they are broken for their existence. As there is less water in the system, this becomes less important and at some level of replacement, these groups are at a lower energy level when in contact with the solvent.

The structure of the protein will be changed and hence, it will be denatured. The reversibility of the process depends to a large extent on the nature of the non-polar solvent, the extent of unfolding the temperature of the system and the rate of solvent removal. When large amounts of the solvent are presented, the protein will be largely unfolded with extensive exposure of the hydrophobic groups. If the protein could be instantaneously transferred to pure water at room temperature, the protein would most likely aggregate and precipitate. The sudden exposure of the hydrophobic groups to water would cause them to try to remove themselves from the aqueous phase as soon as possible. Even before the short range interactions could redirect the folding of the protein aggregation would occur.

If the solvent exchange was slow, there would be a better chance that the hydrophobic groups would be able to return to the interior of the molecule and prevent aggregation. If the exchange occurred at low temperatures, the chances of regaining the native structure would be even better. At low temperatures, the hydrophobic groups may in part be stable in the aqueous phase or at least not as unstable. In this case, the removal of the solvent has little effect. When the temperature is subsequently increased, the normal course of protein refolding can occur. Solvent precipitation is often utilized as a means of purifying and concentrating enzymes. It is extremely important that both the solvent and the protein solution have to be cold when they are mixed and that the subsequent removal of the solvent has to be performed at reduced temperature [24].

2.4.4 Denaturation at interfaces

When proteins are exposed to either liquid-air or liquid-liquid interfaces, denaturation can occur. As a liquid-liquid interface, the protein comes into contact with a hydrophobic environment. If allowed to remain at this interface for a period of time proteins will tend to unfold and place as many of their hydrophobic groups as possible in the non-aqueous layer while maintaining as much charge as possible in the water layer.

Proteins unfold at hydrophobic interfaces, because the tertiary structure of a protein is not rigid. There are continued fluctuations about an average configuration. Any change in conformation that yields a higher energy state will spontaneously go back to the state of lowest energy. As a part of this process, hydrophobic groups will occasionally be positioned so that they have increased contact with the aqueous phase. When this occurs, these groups will assume the configuration of lowest free energy and will be removed from the water. If a hydrophobic group is exposed while a protein is in contact with a polar solvent, these groups will find a state of lower energy exists if they enter into the solvent phase. This will continue to occur until random fluctuations in protein structure can no longer yield a configuration of lower free energy.

The amount of unfolding that occurs at such an interface will depend on how rigid the three-dimensional protein structure is and on the number and location of hydrophobic groups in the molecule. A flexible, non-crosslinked protein will be able to unfold easier than will a highly structured and crosslinked one. If energy is applied to cause shear, the process will be accelerated. The shear can cause the protein to unfold, thus exposing its hydrophobic groups to the nonaqueous phase. It can also increase the interfacial area between the two phases and allow more proteins to come into contact with the nonaqueous phase.

This unfolding is essentially non-reversible because of the large energy barriers. Even if the phases should separate and the protein is forced into the aqueous phase the protein will not

regain its original structure. Rather an association of hydrophobic groups will cause the protein to aggregate.

The same forces are in operation when a protein migrates to a liquid-air interface. Hydrophobic groups tend to associate in the air and the protein unfolds. The presence of shear causes to help unfold the protein and to introduce more air into the solution. Both of these effects can be minimized by keeping the temperature low (to weaken hydrophobic bonds) and by minimizing the interfacial area. If the interface is limited, then only a small amount of protein will be able to denature. The presence of this denatured protein will serve as a barrier to further denaturation. Proteins are often utilized in food products to stabilize emulsions or to incorporate air. These cases will be examined in more detail when emulsions and foams are discussed [24].

2.4.5 Ionic strength

Proteins are usually more soluble in dilute salt solutions than in pure water. The salts are thought to associate with oppositely charge groups in the protein. This combination of charged groups bonds more water than do the charged groups alone and protein hydration is increased. With most proteins there is little change in solubility as more salt is added until some very high salt content is reached. At very high levels of salt there is a competition between the ions and the proteins for water hydration.

When the salt concentration is high enough, the proteins will be sufficiently dehydrated to lose solubility. Removal of the salt or dilution to a low enough concentration will usually result in the recovery of native structure [24].

2.5 Degradation of collagen

2.5.1 Oxidative degradation

Oxidation of collagen was initiated by ozone, ultraviolet light and hydroxyl radicals which were generated with the Fenton reaction or *γ*-irradiation [31].

Oxidative damage involves amino acid modification, increased susceptibility to proteolysis, fragmentation, crosslinking and aggregation. It is implicated in various physiological and pathological processes. Roles for the oxidative destruction of collagens in inflammatory as well as in age-related processes have also been proposed [15].

In addition to the above mentioned oxidation effects, specific for collagen effects, such as depolymerization of the triple helix and impaired ability of the triple helices to assemble into fibrils, have also been considered. Many of the studies focus on collagen fragmentation. Such fragmentation may be occured either as direct result of an oxidation attack, or as a consequence of the enhanced susceptibility of oxidized collagen to enzymatic hydrolysis. The degree of fragmentation depends on the collagen type. Collagen type III is more sensitive and type V is much less sensitive to oxidation than collagen type I. Also, the degradation of acid-soluble collagen is higher than that of fibrillar collagen. One possible route for direct collagen fragmentation is given by the oxidation of proline, followed by cleavage of the Gly-Pro peptide bondw. Except for proline, several other amino acid residues are also sensitive to oxidation attack by metal ion H_2O_2 systems. However, the specific effects of their oxidation on the collagen structure remain at present unclear [15, 31, 32].

Depending on their type and source, collagens experience cooperative thermal denaturational transitions at temperatures typically in the range 10 - 40 °C. It is worth noting that the thermal transitions of the triple helix into a random coil take place closely above the body temperature of the species from which collagen was isolated. Calf skin collagen type I in acidic solution is characterized by a denaturational transition at approximately 41 °C (at commonly used scan rates of 0.5 - 1 °C min). Also, a small endothermic transition at 33 - 35 °C, referred to as “pretransition”, has been recorded in several studies, but the nature of structural changes underlying this transition has not been clarified. Condell et. al. [33] ascribed it for collagen fragments produced during protein isolation at the step of pepsin digestion. It persists though in collagen samples extracted without enzymatic treatment. Bachinger et. al. [34] suggested that the pretransition is caused by unidentified collagen damage during its preparation. Authors noticed that the pretransition was absent in fresh collagen isolated and purified in antioxidant conditions. Oppositely, it was well expressed in collagen solutions stored in aerobic conditions for several months. Since it is conceivable that oxidation may affect the protein thermal stability as well, these observations led them to expect that the pretransition is entirely due to collagen oxidation. They demonstrated that the appearance of an endothermic transition at 35 °C reflects a discrete reduction of the collagen thermal stability at low degrees of oxidation, while more extensive oxidations result in complete disruption of the protein native structure, manifested as abolishment of both denaturational transitions [15].

2.5.1.1 UV degradation

Ultraviolet (UV) radiation is a one of the main components of the electromagnetic radiation. UV radiation in sunlight is divided into three regions dependent on wavelength (λ), UVC (λ=200 - 280 nm), UVB (λ=280 - 320 nm), and UVA (λ=320 - 400 nm). UVC has the highest energy, ε, (ε=c.h/λ, where c is the speed of light in a vacuum, h is Planck's constant) and is the most biologically damaging region of solar radiation [32].

The effects of ultraviolet radiation are both advantageous and deleterious to living organisms. Ultraviolet light has been used since 1877 to sterilise foods, to maintain sterile conditions during surgery, for preventing rickets, for killing pests and insects and for many other purposes. Ultraviolet light absorption is responsible for skin tanning and has been implicated in a causative way in many kinds of skin cancer [35].

The collagen triple helix is sensitive to UV radiation. Electromagnetic (e.g. UV) radiation causes structural damage to collagen, which include the following actions: structural changes of phenylalanine to generate tyrosine (structural scission of OH), decarboxylation (structural scission of C=O), hydrogen abstraction (structural scission of N-H), thermal denaturation, and general oxidative degradation. Any chain scission, which occurs in collagen, has the potential to initiate aging effects or to hasten degradation, leading to changes in collagen bioactivity [32].

2.5.2 Hydrolytic degradation

Hydrolysis of proteins improves their functional, immunological and bioactive properties, making the hydrolysates superior to the native proteins. Functional, bioactive and sensory properties have been shown to be closely related to the degree of hydrolysis (DH) to which the proteins were hydrolysed [36].

Collagen in solution succumbs progresive hydrolitic degradation, which cause loss of many physical properties of collagen. Rate of these proces is depended on temperature, pH of system

and in lesser amout on internal pressure and character of present solvent. Rate of hydrolysis increases with temperature. By neutral pH the degradation advances slowly, the rate increases with movement on both sides. In addition to hydrolysis of transverse covalent bonds which are predominantly esterous, simultaneously a hydrolysis of peptide bonds in polypeptide chain is proceeded. By alkalic hydrolysis this splitting is mild, seventeen times less of peptides are split than esterous bonds. By acidic hydrolysis the split of both types of bonds is approximately identical.

Bromcyan (CNBr) splits the protein molecule at specific locations - at the methionines (in this case towards the C-terminal end). In the collagen molecule, methionine is a relatively rare amino acid (some 10 – 20 amino acids per collagen molecule). The small number of methionine residues leads to a rather limited number of cleavage products (CNBr peptides). The profile of CNBr peptides is typical, at least for the main collagen types, and thus provides an appropriate way to estimate the amount as well as type of collagen in a particular tissue [17].

2.5.3 Enzymatic degradation

Enzymatic hydrolysis has been used for centuries for modification of the functional and nutritional properties of food proteins in the production of traditional foods such as cheeses and fermented plant foods. Enzymatic proteolysis has been shown to increase solubility, modify foaming, emulsifying and gelation properties and to liberate biologically active peptides from certain proteins [37, 38].

The collagenous domain is hardly digested by enzymes other than collagenase due to its stable triple helix but the denatured products such as gelatin and collagen hydrolysate are easily attacked by proteinases [4].

At neutral pH only specific collagenases cleave the native helix at a position about threequarters of the way from the N-terminus. They are zinc containing metalloproteinases of approximately 40 – 50 kDa and generally require calcium as a co-factor for full aktivity. Fibrils as aggregates of collagen molecules are degraded starting from the exterior. Collagenase binds tightly to triple-helices at or near the surface, whereas molecules in the interior become accessible to enzymes in the course of the progressive degradation from the outside. The various types of collagens show different susceptibility to collagenolytic degradation. After the triple-helix is cracked, further degradation of the collagen molecules is facilitated by enzymes such as gelatinases and non-specific proteinases, which cleave the primary fragments into small peptides and amino acids [2].

2.5.3.1 Degradation by collagenases

Collagenases are enzymes that break the peptide bonds in collagen. Collagenases are highly specific proteinases capable of degrading native collagen. Although highly specific, finding assay methods for them has always created problems due mainly to the low solubility of native collagen, which makes distributing equal amounts of substrate difficult since it requires either individual weighings (time consuming) for each tube or rapid distribution of a liquid suspension (inaccurate), as well as the difficulty of distinguishing digestion products from substrate. It is not an exaggregation to say that there are almost as many methods for collagenase assaying as there are groups working in the field [39, 40].

2.5.3.2 Degradation by pepsine

Pepsin is commonly used in preparing soluble collagen. Pepsin-solubilized collagen (PSC) has no N- or C- telopeptides, which are considered to be the antigen sites of collagen. Therefore, PSC is recognized as a potential biomaterial. However, PSC is generally dissolved in weak acid solutions, such as acetic acid, citric acid, etc., and will not dissolve in water, which limits its applications. Proper chemical modification can be a good choice for overcoming this limitation of PSC [41].

2.5.3.1 Degradation by trypsine

Trypsin degraded significantly more pure type I collagen in the pH 4.0 - pretreated group than in the other groups, indicating that pure type I collagen was denatured during acid pretreatment at pH 4.0. The minimal release of native collagen in groups pretreated at pH 5.5 and 7.0 may have been caused by the mechanical action of agitation during the extraction and preparation of pure type I collagen and other experimental procedures, or by the limited digestive effect of trypsin [27].

2.5.3.2 Degradation by cathepsine

Cathepsin K (EC 3.4.22.38) is the predominant cysteine protease and alone is sufficient to completely dissolve insoluble type I collagen.

2.5.4 The effect of protein crosslinkers on denaturation

The presence of groups that crosslink protein molecules will tend to lower the extent of protein denaturation. There are two main reasons that this is so. First, when proteins are crosslinked it is more difficult for them to unfold. As energy is added to the system and secondary bonds are weakened, the presence of crosslinkers will tend to maintain structure. This is especially true if the crosslinks are covalent as in the case of disulfide bonds. The more compact the molecule is and the greater the number of disulfide linkages present, the greater the stability of the protein. While secondary forces may be weakened and some bonds can be broken, the crosslinkers will tend to keep these groups in fairly close proximity. They also tend to prevent the exposure of large numbers of hydrophobic groups to the solvent. When conditions are returned to the native state, there is now a much greater chance for the proper secondary interaction to occur and for the protein to assume the native configuration.

A second effect has to do with the differences in entropy between the native and unfolded states. If a protein can be caused to assume a completely random coil conformation, there will be a large increase in entropy compared to the native structure. This entropy must be overcome if the protein is to refold into a native conformation. When crosslinking groups are present, a completely random coil conformation can not be assumed. These groups introduce order into the structure and there is a considerable loss in the amount of disorder that can be achieved in the most denatured state. Because of this, the entropy change between the native and denatured state is not nearly as great and there will be less of a driving force for denaturation. If the crosslinking groups are broken before denaturation and thus allowed to randomly form after denaturation, no stability will be added to the protein by the pressure of these groups [24].

2.6 Determination of degradation rate

Numerous methods exist for the estimation of degree of hydrolysis (DH). It can be quantified by determining the amount of nitrogen released during hydrolysis, which becomes soluble in the presence of a precipitating agent (e.g. trichloroacetic acid). Methods used include the Kjeldahl method or spectrophotometric determination in the visible region after colorimetric reaction e.g. biuret reaction. DH can also be quantified by determination of the free amino groups released during hydrolysis by formol titration, or by using compounds which react specifically with amino groups such as trinitrobenzenesulphonic acid (TNBS), o-phthaldialdehyde (OPA), ninhydrin or fluorescamine. DH can also be quantified using osmometry, where the depression in freezing point can be used to calculate changes in osmolality during hydrolysis, which is then used to calculate DH. Alternatively, in the pH stat method the protons released during hydrolysis are titrated and then related to DH [38].

2.6.1 PH stat method

The principle of the pH stat technique is that when hydrolysis is carried out at neutral or alkaline conditions, dissociation of protons is favoured from the free amino groups released. The liberation of protons into the surrounding medium leads to a reduction in the pH of the reaction mixture. The number of peptide bonds cleaved can be estimated from the amount of base required to maintain a constant pH during the reaction. The percentage DH is calculated using the following formula (6) [44].

$$DH\% = 100 \cdot B \cdot N_b \cdot \left(\frac{1}{\alpha}\right) \cdot \left(1 \cdot (MP) \cdot \left(\frac{1}{h_{tot}}\right)\right) \quad (6)$$

Where B is the base consumption in mL, N_b the normality of the base, α the average degree of dissociation of the α-NH_2 groups, MP the mass of protein being hydrolysed (g), and h_{tot} the total number of peptide bonds in the protein substrate (meqv.g^{-1} protein) [38]. The degree of dissociation (α) for the α-NH_2 groups was calculated as follows (7).

$$\alpha = \frac{(10 \cdot (pH - pK))}{(1 + 10(pH - pK))} \quad (7)$$

Where pK is the average dissociation value for the amino groups liberated during hydrolysis and is dependant on temperature, peptide chain length and the nature of the terminal amino acid. The parameter h_{tot} is given as meqv peptide bonds per gram of protein. This was calculated from amino acid analysis by summing the mmoles of each individual amino acid per gram of protein.

The pH stat method was found to be a quick and simple method for quantifying the *DH*. The method is non-denaturing and allows for real-time monitoring of the hydrolysis reaction as it proceeds. However, the accuracy of *DH* values obtained by the pH stat method depends on the type of enzyme activity used in the hydrolysis reaction. When the enzyme preparation is rich in exopeptidase activities the pH stat method under-estimates *DH* [38].

2.6.2 Radioactiv marking

Methods utilizing radio-labelled collagens are considered more convenient, since they are very sensitive and the amount of label released can be immediately determined. Radiolabelling with ^{14}C, ^{18}O or with ^{2}H or ^{3}H, however, requires expensive devices for registering the released marker and moreover, it leads to the generation of radioactiv waste [45, 46].

2.6.2.1 ^{14}C-labelling

The method is based on incorporating [^{14}C]proline and [^{14}C]lysine into collagen as [^{14}C]hydroxyproline and [^{14}C]hydroxylysine at a constant rate for at least 24 h [46]. The samples are lyophilized to dryness, hydrolyzed (in HCl), decolorized, filtered, and evaporated to dryness. Quantitation of [^{14}C]hydroxyproline and [^{14}C]hydroxylysine is made by ion exchange chromatography. Prior to being applied to the column the samples are redissolved (pH 2.2 for [^{14}C]hydroxyproline and 4.25 for [^{14}C]hydroxylysine), and filtered. Fractions of the column effluent are collected in counting vials and 5 ml of Aquasol is added to each one (counting efficiency, 80 %). The quantity of [^{14}C]hydroxyproline and [^{14}C]hydroxylysine eluting from the column is determined by summing the fractions making up the radioactive peak [46].

Finding small peptides containing labeled hydroxyproline represents degradation of the collagen *α*-chain, not only the NH_2-terminal precursor piece of procollagen [46].

2.6.2.2 ^{18}O-labeled

The use of gas chromatography-mass spectrometry (GC-MS) and $^{18}O_2$, a stable isotope, which is incorporated into collagen during the post-translational conversion of proline to hydroxyproline, offers the potential advantages of high levels of sensitivity and specificity as compared to other techniques for measuring rates of collagen synthesis and degradation *in vitro* and *in vivo.* Trifluoracetylation and methanol esterification of hydroxyproline yields two derivatives of hydroxyproline: N,O-trifluoroacetyl methyl 4-hydroxy-L-proline (N,O-TFA-Hyp) and N-trifluoroacetyl methyl 4hydroxy-*L*-proline (N-TFA-Hyp). By labelling fibroblasts in culture with $^{18}O_2$, a sample of isotope-enriched collagen is obtained which is used to calibrate the GC-MS over the range 0.5 – 49.0 % atom percent enrichment (APE) [45].

As a tracer for collagen metabolism, $^{18}O_2$ offers the advantages of a non-recycling label uniquely incorporated at a post-translational level, for which the precursor pool size is small and rapidly turning over and whose product 4-hydroxy-*L*-proline (Hyp) is almost unique to collagen and critical for the formation of a stable triple helix [45].

2.6.3 Immunological method

Immunological methods for determination the degradation of collagen are sensitive and specific, but they require equipment not always available in the biochemistry laboratory [39].

2.6.4 Determination of the nitrogen amount

2.6.4.1 Biuret reaction

The method is based on spectrophotometric determination in the visible region after colorimetric reaction. Peptides, proteins and amino acids react with copper sulfate in highly

alkaline solutions to produce a blue-violet colored chelate complex (Fig. 7). This complex has an absorbance maximum at 540 nm. The peptide must have three peptide bonds at least.

The biuret test is used for the quantitative photometrical determination of total protein concentration. The intensity of the color produced in the biuret reaction is proportional to the number of peptide bonds participating in the reaction [42].

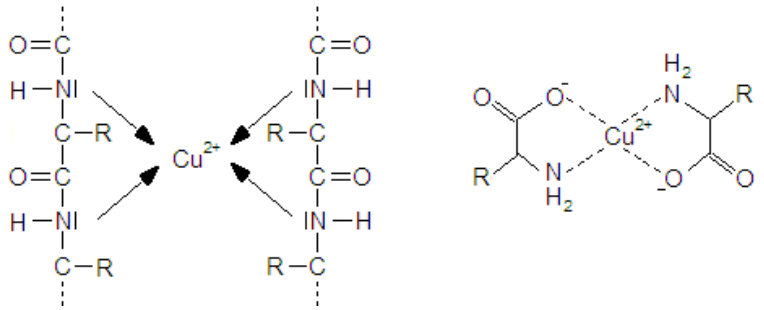


Fig. 7 *Complex create reaction peptides, proteins and amino acids with Cu^{2+} [42].*

2.6.4.2 Kjeldahl method

The Kjeldahl method was developed over 100 years ago for determining the nitrogen contents in organic and inorganic substances.

Kjeldahl nitrogen determinations are performed on a variety of substances such as meat, feed, grain, waste water, soil, and many other samples. Various scientific associations approve and have refined the Kjeldahl method, including the AOAC, International Association of American Cereal Chemists, American Oil Chemists Society, Environmental Protection Agency, International Standards Organization, and United States Department of Agriculture.

The Kjeldahl method may be broken down into three main steps: digestion, distillation, and titration [43].

Digestion is accomplished by boiling a homogeneous sample in concentrated sulfuric acid. The end result is an ammonium sulfate solution. In the distillation step an excess of base is added to the digestion product to convert NH_4 to NH_3 as indicated in the equation (2).

$$(NH_4)_2SO_4 + 2\,NaOH \xrightarrow{\text{heat}} 2\,NH_3(g) + Na_2SO_4 + 2\,H_2O \qquad (2)$$

The NH_3 is recovered by distilling the reaction product. Titration quantifies the amount of ammonia in the receiving solution. The amount of nitrogen in a sample can be calculated from the quantified amount of ammonia ion in the receiving solution.

There are two types of titration - back titration and direct titration. Both methods indicate the ammonia present in the distillate with a color change [43]. In back titration (commonly used in macro Kjeldahl), the ammonia is captured by a carefully measured excess of a standardized acid solution in the receiving flask. The excess of acid in the receiving solution keeps the pH low, and the indicator does not change until the solution is "back titrated" with base (3) and (4).

$$2\,NH_3 + 2\,H_2SO_4 \rightarrow (NH_4)_2SO_4 + H_2SO_4 \qquad (3)$$
(no color change)

$$(NH_4)_2SO_4 + H_2SO_4 + 2\,NaOH \rightarrow Na_2SO_4 + (NH_4)_2SO_4 + 2\,H_2O \quad (4)$$
(color change occurs)

In direct titration, if boric acid is used as the receiving solution instead of a standardized mineral acid, the chemical reaction is represented by (5).

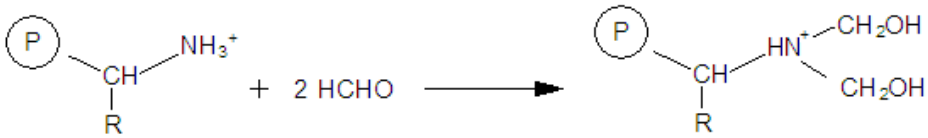
$$NH_3 + H_3BO_3 \rightarrow NH_4 + H_2BO^-_3 + H_3BO_3\ (excess) \quad (5)$$
(color change occurs)

The boric acid captures the ammonia gas, forming an ammonium-borate complex ($NH_4 + H_2BO^-_3$). As the ammonia collects, the color of the receiving solutions changes [43].

2.6.5 Determination of the free amino group

2.6.5.1 Formol titration

Formol titration is a simple, quick and reliable method of determining the amino groups of amino acids by adding formaldehyde to the neutral solution. Addition of formaldehyde forms more acidic ternary amino groups by the reaction between primary amino groups and formaldehyde (8).



(8)

The formaldehyde reacts with the NH^{3+} group, liberating an equivalent quantity of H^+, which may then be estimated by titration with NaOH to a selected pH end point. The added alkali is taken to be aquivalent to the amino groups present initially in the protein.

The nature of the N-terminal amino acids in sample can vary according to the specificity of the method used for the hydrolysis and to the stability of peptide bonds involved.

The initial and final pH selected to carry out the formol titration must be adapted to the dissociation constant (pK) of the different entities [41, 47].

The result of the formol titration is N_t, the total free amino groups present initially in sample. To obtain the N-terminal amino acids N_α (α-amino groups), the N-lateral amino acids N_ε (ε-amino groups) must be substracted (9).

$$N_\alpha = N_t - N_\varepsilon \quad (9)$$

2.6.5.2 Fluorescent methods

Fluorescent collagens look more attractive, but labelling with fluorescein isothiocyanate (FITC) in Fig. 8 for instance is time-consuming due to the procedures required separation from unbounded marker resulting in low yield [39].

Although a number of different types of fluorescent tagging reagents have been developed, many reports have described various shortcomings in application: o-phthaldialdehyde (OPA); 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole for primary and secondary amino compounds; 2-(9-Fluorenyl)-ethyl chloroformate (FMOC), 1-(9-fluorenyl)-ethyl chloroformate, and 2-(9-anthryl)-ethyl chloroformate (AEOC) for the derivatization of amino acids and peptides for chiral or non-chiral separation in LC or CE; 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) or 2-(9-carbazole)-ethyl chloroformate (CEOC) [48].

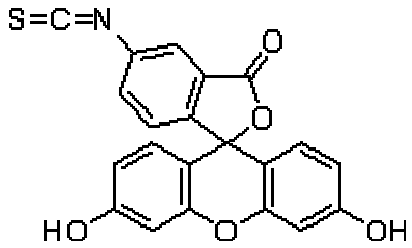


Fig. 8 *Fluorescein isothiocyanate (FITC).*

OPA (O-phthalaldehyde)

OPA (Fig. 9) is used for precolumn derivatization of amino acids for HPLC separation and for flow cytometric measurements of protein thiol groups [49].

The o-phthaldialdehyde method offers greater sensitivity, more selectivity, quick and simple method but is limited to primary amino acids only. This method does not involve long incubation steps, which again allows for real-time measurements. The OPA method is based on the specific reaction between OPA and primary amino groups, in the presence of a thiol to form 1-alkylthio-2-alkyl-substituted isoindoles. The formed isoindoles can be quantified spectrophotometrically at 340 nm or fluorometrically at 455 nm [36, 48].

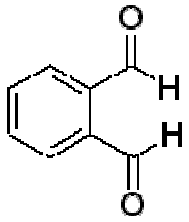


Fig. 9 *OPA (O-phthalaldehyde).*

2.6.5.3 Ninhydrin

Ninhydrin is used for the detection of free amino groups in amino acids, peptides and proteins. The ninhydrin assay also has been used to determine the extent of protein hydrolysis [36]. Amines (including *α*-amino acids) react with ninhydrin to give a coloured product (Fig. 10). This reaction can be used qualitatively (e.g. for chromatographic visualisation) or quantitatively (e.g. for peptide sequencing). With *α*-amino acids ninhydrine give a tyically blue-purple product and with secondary amine (proline) give a yellow-orange product [50].

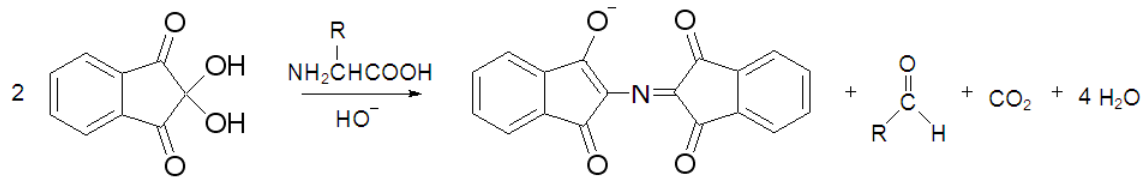


Fig. 10 *Ninhydrin test.*

2.6.5.4 Fluorescamine (4-Phenylspiro-[furan-2(3H),1-phthalan)]-3,3'-dione

Fluorescamine (Fig. 11) is non-fluorescent reagent that reacts readily under mild conditions with primary amines in amino acids and peptides to form stable, highly fluorescent compounds.The method is useful for the fluorometric assay of amino acids, protein, and proteolytic enzymes. Moreover, fluorescamin effectively blocks newly generated amino termini in protein sequence analyses [49].

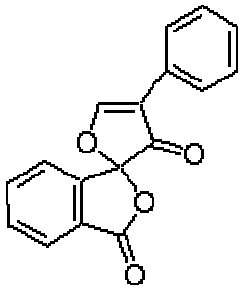


Fig. 11 *(4-Phenylspiro-[furan-2(3H),1-phthalan)]-3,3'-dione (fluorescamine).*

2.6.5.5 Spectroscopy method using FDBN, CITNB and TNBS

The amount of ε-amino group present in the protein after reaction with reagent is directly quantitated using the standard curve generated by the difference in absorbance.

FDBN

The FDBN (1-fluor-2,4-dinitrobenzen) method of Sanger has been used extensively in past. Not for the N-terminal analysis of peptides and proteins, but also for an assay or identification of aminoacids, -alcohols, -sugars, -lipids. Optimal conditions for trinitrophenylation of protein (TNP-lation) with FDBN are 66 % of alcoholic solutions at pH from 5 to 8 and room temperature. This method determines the available lysine content of protein and observed a good correlation between the available lysine content and the biological values of protein. However, the FDBN method is laborious and time consuming for the routine analysis of proteins [9, 28, 51].

CITNB

The CITNB (1-chlor-2,4,6-trinitrobenzen) was introduced into the amino group of amino acid or lower peptides and partially into water molecule. Reaction conditions are identical with conditions for TNP-lation with FDBN [39, 52]. Evaluation of the reaction mixture is done by means of paper chromatography.

TNBS

The TNBS (2,4,6-trinitrobenzen sulfonic acid) (Fig. 12) method is a sensitive method for determine the free amino group in proteins. However, the TNBS method requires long incubation and cooling steps (1 h and 30 min, respectively) with the result that the assay cannot be used for real-time monitoring of DH [38].

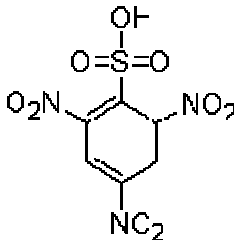


Fig. 12 *2,4,6-trinitrobenzen sulfonic acid (TNBS).*

TNBS reacts specifically with primary amino groups to form a chromophore with a maximum absorbance at 340 nm (

Fig. **13**) [53]. This reaction is pH-dependent. Maximum absorbance advance is at pH 8.5 or 9 and this is dependent on the particular protein investigated [54].

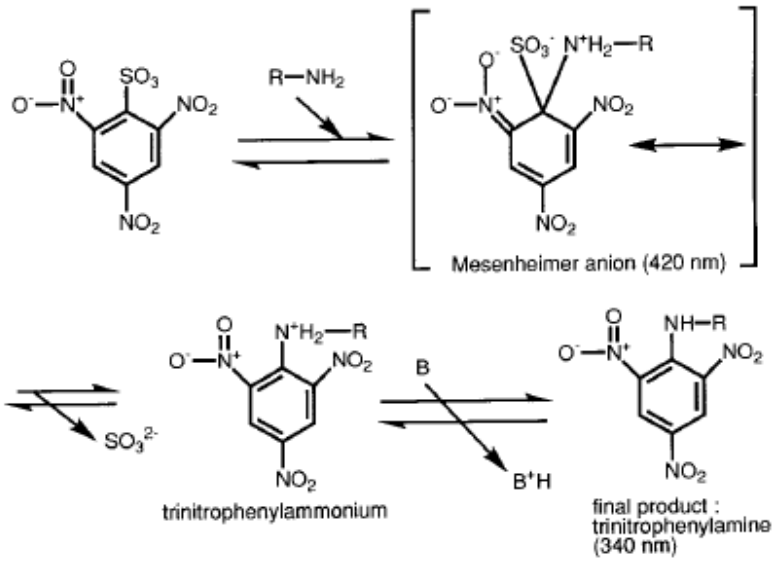


Fig. 13 *Trinitrophenylation mechanism [55].*

In the evaluation of the sensitivity, complexity and execution time performance of the above methods spectrophotometric method are selected owing to sufficient sensitivity and simplicity. As the best reagent 2,4,6-trinitrobenzensulfonic acid and ninhydrin as comparative agent was chosen in the experimental part of the presented work.

3 EXPERIMENTAL PART

3.1 Chemicals

- Collagen type I, 8% aqueous solution (VUP a.s., CZ)
- 2,4,6-trinitrobenzenesulfonic acid solution, TNBS, 5 % (w/v) in H_2O (Aldrich Chemical Company, Germany)
- 2,2-Dihydroxy-1,3-indanedione, Ninhydrin (Aldrich Chemical Company, Germany)
- *L*-lysine (Aldrich Chemical Company, Germany)
- Sodium dodecyl sulfate, 10% (Penta, CZ)
- Hydrochloric acid, 1 M (Lach-Ner, s.r.o., CZ)
- Ethanol 98%. (Lach-Ner, s.r.o., CZ.)
- Sodium bicarbonate (Lach-Ner , s.r.o., CZ)
- Potassium bromide (Lach-Ner , s.r.o., CZ)

3.2 Equipments

- Analytical scales (Mettler Toledo classic AB204S)
- Lyophilizator (LABCONCO, Freeze Dry System/Freezone 4.5)
- Disintegrator (IKA Turrax basic T18. D)
- pH meter (Hanna Instruments 211)
- Centrifuge (Hettich EBA 30. D)
- Air-drier (Hydraflow Snackmaker FD 500)
- UV-VIS Spectrophotometer (Helios Alpha Unicam)
- IR spectrometer (Nicolet iso10)
- Scanning electron microscope (FEI Quanta 200 Mk2)

3.3 Collagen samples

3.3.1 Preparation of samples

An aqueous collagen solution (8 wt%) was used for the preparation of collagen samples. 0.2 wt% collagen solutions were prepared by swelling 5 g of 8 wt% collagen solution in 195 ml of distilled water. The prepared collagen solution (0.2 wt%) was physically or chemically modified (see 3.3.1.1). Modified collagen solutions were homogenized by disintegrator (for 3 min at 14 000 rpm, except of set B) and placed in polystyrene Petri dishes, froze at -30 °C for 24 hours and then lyophilized at -80 °C and pressure of 15 Pa. Required quantity of obtained 100% collagen lyophilisate was swelled in distilled water in order to prepare concentration of 3 $mg.ml^{-1}$ for analysis.

3.3.1.1 Modification of samples

Collagen samples were subjected to various modifications (see Tab 6 - 9) and then compared the effect of modifications on the quantity of free aminogroups in the collagenous samples. Sample modifications were done before freezing and lyophilization followed by spectroscopy analysis.

Collagen samples were exposed by four types of modifications. Collagen samples without modification were termed as set A (reference sample). Samples modified by different time (1 - 5 min) and intenzity (6 000 -14 000 rpm) of disintegration at three different temperatures (4 °C, lab. temp. and 30 °C) were termed as set B (Tab. 6). Samples treated by different processing time proceeded from the preparation till freezing upon three different temperatures (4 °C, lab. temp. and 30 °C) in dark (protected by aluminum foil) were termed as set C (Tab. 7). Samples treated by different processing time proceeded from the preparation till freezing at light were termed as set D (Tab. 8) and last set E represent samples modified by different value of pH (Tab. 9). Adjustments of pH were carried out by addition of sodium bicarbonate buffer. Laboratory temperature was 22 °C. Samples of set C, D and E were disintegrated at laboratory temperature for 3 min at 14 000 rpm.

Tab. 6 *Samples of set B – modification by different intenzity of disintegration and temperature*.

Sample	Temperature	Time of disintegration	Intenzity of disintegration
B1	4 °C	1 min.	6 000 rpm
B2	4 °C	2 min.	10 000 rpm
B3	4 °C	3 min.	14 000 rpm
B4	4 °C	4 min.	14 000 rpm
B5	4 °C	5 min.	14 000 rpm
B6	lab.	1 min.	6 000 rpm
B7	lab.	2 min.	10 000 rpm
B8	lab.	3 min.	14 000 rpm
B9	lab.	4 min.	14 000 rpm
B10	lab.	5 min.	14 000 rpm
B11	30 °C	1 min.	6 000 rpm
B12	30 °C	2 min.	10 000 rpm
B13	30 °C	3 min.	14 000 rpm
B14	30 °C	4 min.	14 000 rpm
B15	30 °C	5 min.	14 000 rpm

Tab. 7 *Samples of set C – modification by different time from preparation till freezing upon different temperatures in dark. Samples were disintegrated for 3 min at 14 000 rpm.*

Sample	Temperature	Time from preparation till freezing (day)
C1	4 °C	1
C2	4 °C	2
C3	4 °C	3
C4	4 °C	4
C5	4 °C	5
C6	lab.	1
C7	lab.	2
C8	lab.	3
C9	lab.	4
C10	lab.	5
C11	30 °C	1
C12	30 °C	2
C13	30 °C	3
C14	30 °C	4
C15	30 °C	5

Tab. 8 *Samples of set D – modification by different time from preparation till freezing at light. Samples were disintegrated for 3 min at 14 000 rpm.*

Sample	Temperature	Time from preparation till freezing (day)
D1	lab.	1
D2	lab.	2
D3	lab.	3
D4	lab.	4
D5	lab.	5
D6	lab.	8
D7	lab.	9
D8	lab.	10
D9	lab.	11
D10	lab.	14

Tab. 9 *Samples of set E – modification by different value of pH*. Samples were disintegrated for 3 min at 14 000 rpm.*

Sample	Temperature	Value of pH
E1	lab.	4
E2	lab.	5
E3	lab.	6
E4	lab.	7
E5	lab.	8

*value of pH was modified by addition of $NaHCO_3$

3.3.2 Collagen films

As for collagen films preparation, 5 ml of homogeneous colagen solution (3 $mg.ml^{-1}$) of samples modified according to sets B, C and D were placed in polystyrene Petri dishes with 5 cm diameter in order to create a film of suitable thickness. In case of set E, collagen solutions were partly separated from water due to the pH adjusting, thus higher quantity of solution (10 ml) had to be used. Prepared samples in Petri dishes were dried to the constant weight in air-drier for 48 hrs.

3.4 Characterizaton of samples

The chemical, physical and mechanical treatment of the collagen solution might cause disruption in collagen structure. The extent of disruption in the collagen natural structure can be specified by determining the amount of free amino groups, which correspond to breaking in intermolecular links at the collagen quaternary structure.

Changes in the collagen structure were assessed on the basis of UV-VIS and FT-IR spectroscopy together with comparing images obtained from scanning electron microscopy (SEM).

3.4.1 UV-VIS spectroscopy

Free amino-groups presented in the collagen can be modified by adding a suitable reagent (in this case ninhydrin or TNBS) that provides a colored pruduct after the reaction with free amino group. Quantity of the colored product can be determined using UV-VIS spectroscopy. The evaluation of samples were done using a method of calibration curves (see 3.4.1.1), where *L*-lysine solution at a concentration of 0.001 - 0.01 $mg.ml^{-1}$ was used as a standard solution. Concentration of free amino groups in the modified collagenous samples was calculated using a regression equation of the calibration curve. Concentration of free amino groups was converted to percentages on the basis of aminoacid analysis of the collagen. Amino acid analysis was carried out at Veterinary and Pharmaceutical faculty of Brno by acid hydrolysis for 24 hours at 110 °C.

3.4.1.1 Calibration curves

$2.5 \cdot 10^{-3}$ g of *L*-lysine was dissoved in 250 ml of distilled water resulting in 0.01 $mg.ml^{-1}$ concentration of *L*-lysine solution. Prepared *L*-lysine solution was used for a calibration curve adjusting (Tab. 10).

Tab. 10 *Preparation of calibration curve of L-lysine solution.*

Concentration [$mg\text{-}ml^{-1}$]	Volume of *L*-lysine solutions of concentration 0.01 $mg.ml^{-1}$ [ml]	Volume of distilled water [ml]
0.001	0.2	1.8
0.002	0.4	1.6
0.003	0.6	1.4
0.004	0.8	1.2
0.005	1.0	1.0
0.006	1.2	0.8
0.007	1.4	0.6
0.008	1.6	0.4
0.009	1.8	0.2
0.010	2.0	0.0

2 ml of prepared *L*-lysine solution was placed into 40 ml of brown glass vials. In case of reaction with TNBS 2 ml of 0.1 M $NaHCO_3$ solution was added and solution was stirred. Then 1 ml of fresh prepared reagent (0.01 % aqueous solution of TNBS) was added and solution was mixed again. Vials with the reaction mixtures were fitted with screw cap and placed in a water bath at 40 °C for 2 hours. After this period, vials were removed and 1 ml of 10 % solution of sodiumdodecyl sulfate (SDS) was added to reduce clot formation and solution was shuffle. Then the reaction was terminated by adding 0.5 ml of 1 M HCl and absorbance of yelow product was measured at a wavelength of 340 nm followed by calibration curve evaluating.

In case of reaction with ninhydrine 1 ml of fresh prepared reagent (0.2% alcohol solution of ninhydrine) was added and solution was mixed. Vials with the reaction mixtures were fitted with screw cap and placed in boiling water bath for a period of 20 minutes. After this period, vials were removed and cooled down. 9 ml of distilled water was subsequently added due to the overranged absorbance of nondiluted solution. The absorbance of blue-purple product was measured at a wavelength of 570 nm followed by calibration curve evaluating.

3.4.1.2 TNBS

2,4,6-trinitrobenzensulfonic acid (TNBS) is frequently used agent in determining the proteins. TNBS reacts with primary amino groups to produce colored product, whose absorption maximum is around 340 nm. The reaction carried out for 2 hours at 40 °C and at slightly alkaline pH. Since the reagent is not stable on the light the reaction must be avoided to contact with light. For each analysis freshly prepared solution of TNBS was used.

Into 40 ml vials of brown glass 2 ml of collagen solution having concentration of 3 $mg.ml^{-1}$ were placed. Then the samples were treated same way as in case of the calibration curve (see 3.4.1.1). For each sample the procedure was repeated 5 times. After termination solutions were

transferred to centrifugation tubes and separated at 5 000 rpm for 9 minutes. Subsequently the absorbance was measured at wavelength of 340 nm against a blank sample.

3.4.1.3 Ninhydrin

Ninhydrin (2,2-dihydroxy-1,3-indanedione) forms colored products with amino groups contained in the proteins. With *α*-amino acids ninhydrin gives a tyically blue-purple product, whose absorption maximum lies at 570 nm. Ninhydrin solution was prepared in alcohol and because of its instability it should be kept in the dark and prepared fresh prior to use. The reaction of ninhydrin with the free amino groups proceeded via boiling reaction mixture.

Into 40 ml vials of brown glass 2 ml of collagen solution having concentration of 3 $mg.ml^{-1}$ were placed. Then the samples were treated same way as in case of the calibration curve (see 3.4.1.1). For each sample the procedure was repeated 5 times. The samples of set E had to be centrifugated because of clot presence. Absorbance of the samples was measured at wavelength of 570 nm against the blank sample.

3.4.2 FT-IR spectroscopy

FT-IR analysis of selected samples was conducted for the qualitative determination of bonds presented in collagen samples. Samples were measured in the form of tablets or films. IR spectrometer Nicolet iso10 evaluated at 64 scans was used. Obtained spectrum was adjusted by Omnic program.

Tablets were prepared from reaction products of collagen samples with TNBS, which were dried in air-drier. Accurately weighted quantity (20 mg) of obtained powder was mixed with 100 mg of potassium bromide and tablets were pressed at a pressure of approximately 200 kPa. During the pressing so-called “glassy modification“ of patassium bromide was created in order to loose only small intenzity of infrared radiation.

Selected prepared films (3.3.2) were measured by transmission method.

Due to the very high amount of samples, which had to be measured at least 5 times, only selected ones were measured usinf FT-IR. Only films of samples B1, B2, B3, B5, B6, B7, B8, B10, B11, C1, C2, C3, C4, C5, C6, C7, C8, C9, C10, C12, C13, C15, D2, D4, D6, D8, D10, E1, E2, E3, E4 and E5 were evaluated.

3.4.3 Scanning electron microscopy

Scanning electron microscope (SEM) FEI Quanta 200 Mk2 was used to investigate morphology of selected collagen samples. Images obtained from lyophilisated scaffolds cross sections were compared with a view of porosity, pore size and structure.

4 RESULTS AND DISCUSSION

4.1 UV-VIS spectroscopy

Aqueous solution of 2,4,6-trinitrobenzensulfonic acid (TNBS) and ninhydrin solution in ethanol were used as reagents for UV-VIS analysis of the free amino groups amount of modified collagen solutions. Due to the nonhomogeneous structure of collagen water solutions, the free amino groups amount was not considered in absolut values. Better information gives the trend of values for each collagen modification of individual sets, which were monitored and compared.

4.1.1 Calibration curve

The calibration curves of *L*-lysine reaction with both TNBS (Fig. 14) and ninhydrin (Fig. 15) reagent were assessed from absorbance values of colored reactions with free amino groups.,. Regression equation of each calibration curve was used for free amino groups' content calculation.

Becaused of a different pH of collagen solution in comparison with *L*-lysine solution the calibration curve was performed with adjusted value of pH as well. Product of the reaction with adjusted pH had a yellow-brown color, which was formed probably from the mixture of blue-purple product of ninhydrine reaction with *α*-amino acids and yellow-orange product of ninhydrine reaction with secondary amine. Therefore the calibration curve was performed without adjusted pH.

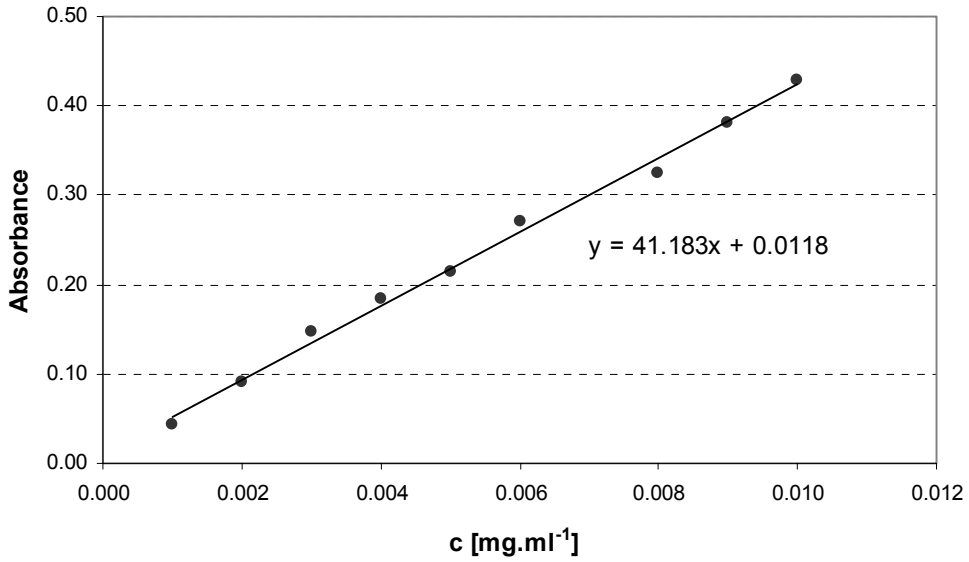


Fig. 14 *Calibration curve of L-lysine after the reaction with TNBS.*

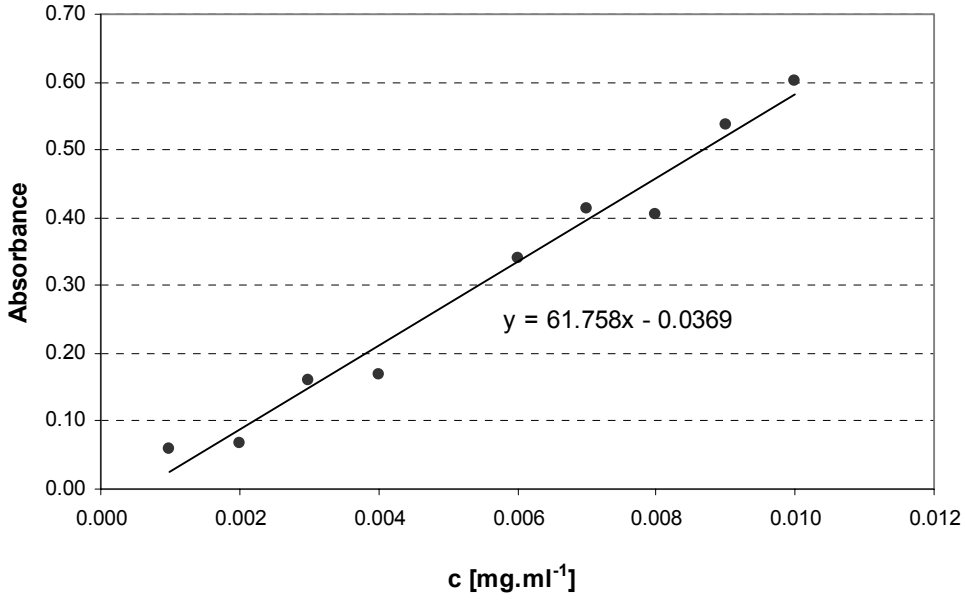


Fig. 15 *Calibration curve of L-lysine after the reaction with ninhydrine.*

4.1.2 Influence of intensity and time of disintegration

Contents of free amino groups in collagen samples of set B after reaction with TNBS ranging from 2 to 5 % (tab. A 1 in appendix).

Taking into account the standard deviations, values of samples disintegrated at the same temperature showing, in most cases, only small differences regardless the time and intensity of disintegration as can be seen from Fig. 16 and Fig. 17, respectively. Higher differencies in content of free amino groups occur when comparing samples prepared at different temperatures. Surprisingly, the highest amount of free amino groups were obtained when disintegration proceeded up to 4 min at lab. temperature. The same trend was recorded for values obtained after reaction with both TNBS and ninhydrin. However, the content of free amino group obtained by reaction with ninhydrin in comparison with TNBS is significantly lower. The value of free amino groups content is for most samples around 0.6% with standard deviation 0.097 (tab. A 1 in appendix). The difference between the values obtained using TNBS and ninhydrin is probably due to variant sensitivity of the reagents.

Therefore, the time and intensity of disintegration in comparison with the influence of temperature plays insignificant role in the emergence of free amino group.

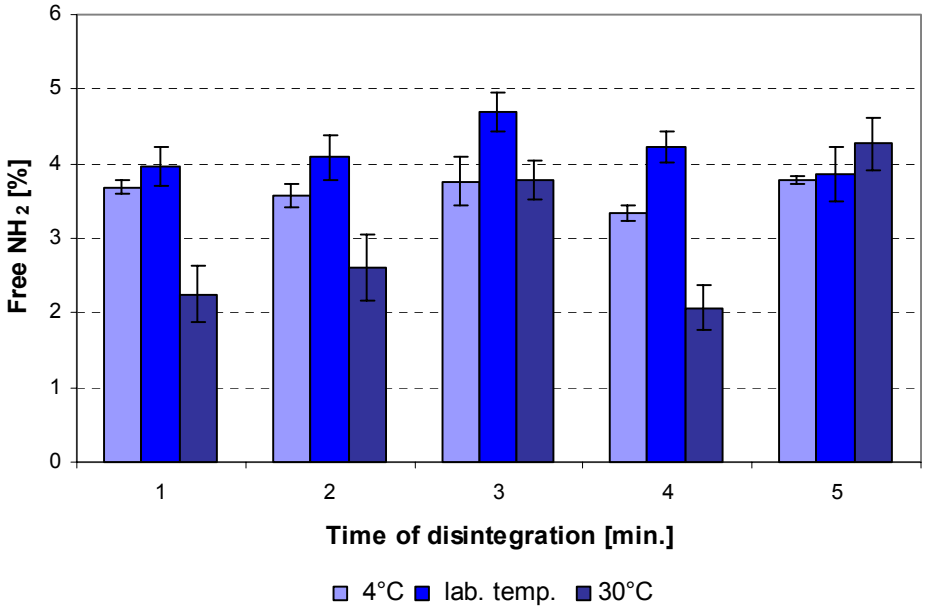


Fig. 16 *Content of free amino groups by TNBS in collagen samples modified by time and temperature of disintegration.*

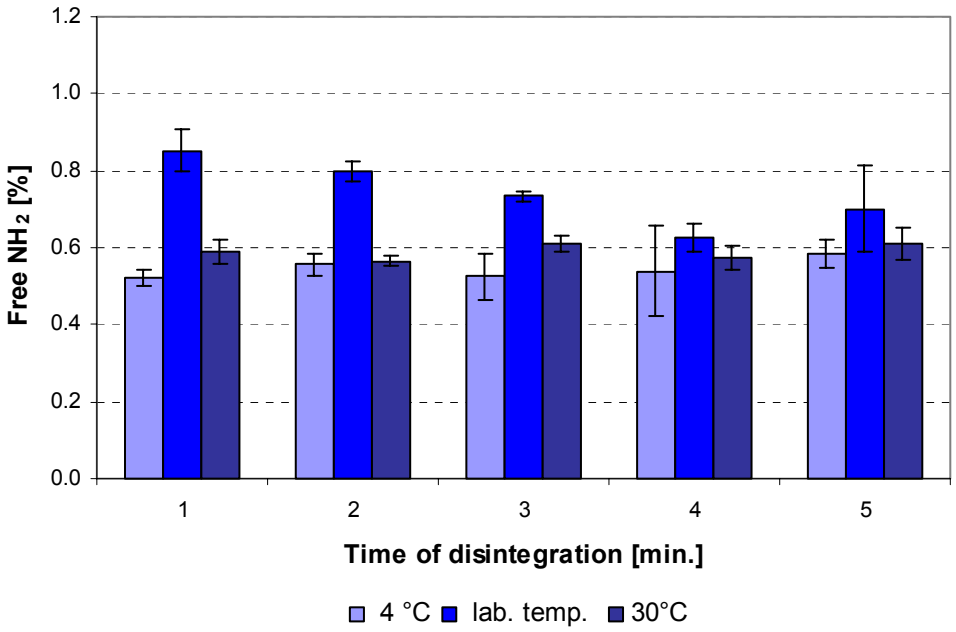


Fig. 17 *Content of free amino groups by ninhydrine in collagen samples modified by time and temperature of disintegration. Influence of time and temperature of preparation.*

In the case of samples treated by different processing time proceeded from the preparation till freezing upon three different temperatures (4 °C, lab. temp. and 30 °C) in dark (set C) amount of free amino groups increases with time from preparation until freezing (Tab. A 2 in appendix) and with growing temperature after the reaction with TNBS. While there were almost no difference between the temperatures of preparation of 4 °C and lab. temp., the most significant increase in the amount of free amino groups arised at 30 °C, particularly when preparation proceeded longer than 1 day (Fig. 18). The results confirms the theoretical assumption that growing temperature and time of collagen samples preparation cause a changes in the structure of the sample accompanied by release of amino groups.

Regarding the data obtained from the reaction with ninhydrin the values of free amino groups were more stable but slightly higher comparing with reference sapmle of set A (Fig. 19). Samples showed the amount of about 0.6 % as in the case of samples modified by different time and intenzity of disintegration (set B).

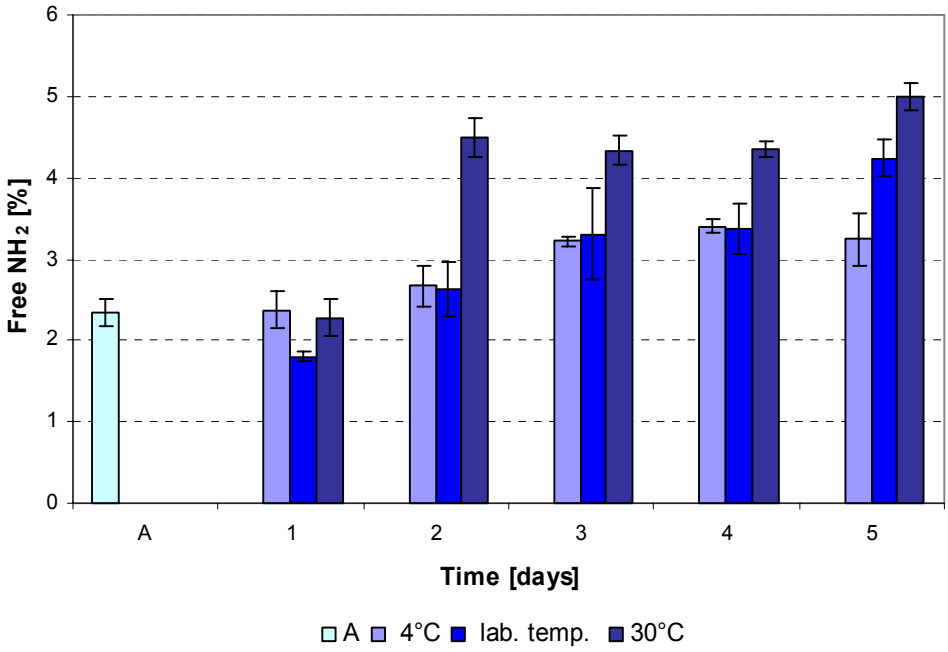


Fig. 18 *Content of free amino groups in collagen samples modified by increasing time of preparation at different temperatures, measured after the reaction with TNBS anfd protected from light.*

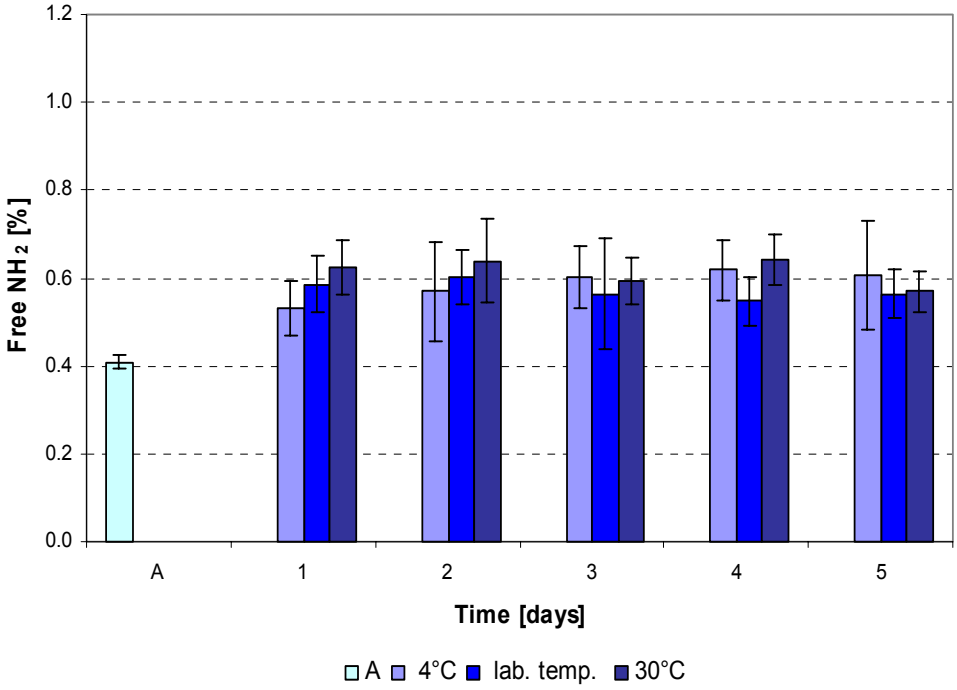


Fig. 19 *Content of free amino groups in collagen samples modified by increasing time of preparation at different temperatures, measured after the reaction with ninhydrine and protected from light.*

4.1.3 Influence of light duration

As for samples, which were left at laboratory temperature on the light for a different periods of time (set D), an increase in the amount of free amino groups with light duration was observed (tab. A 3 in appendix). The increase is more noticeable at the values measured after the reaction with TNBS (Fig. 20) than in the case of the values measured after the reaction with ninhydrin (Fig. 21). Moreover, increasing light duration made changes in samples consistency. Within first days of the sample's preparation on the light a homogeneous gel was formed, which later on agglomerated and sample became poorly liquid.

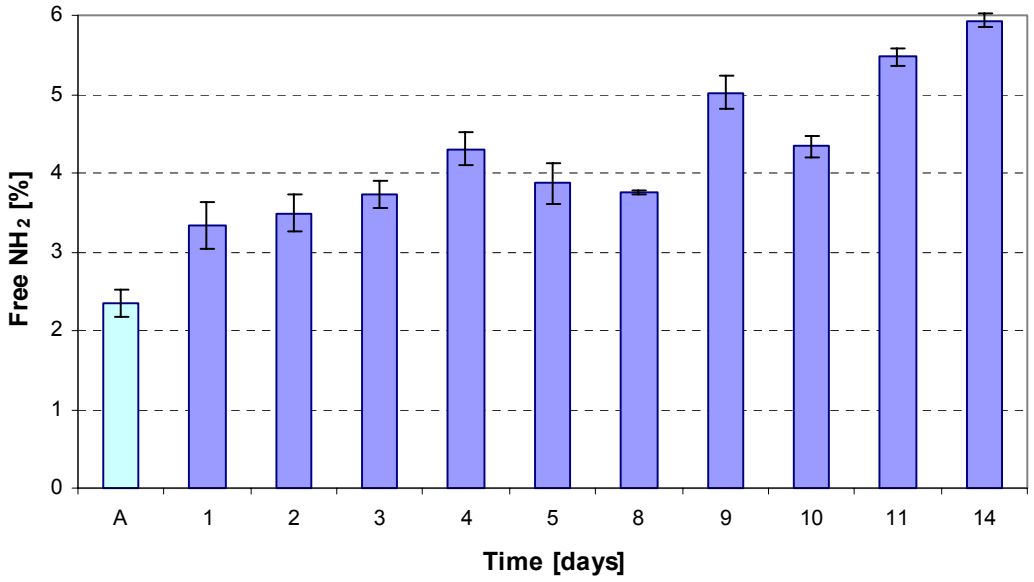


Fig. 20 *Content of free amino groups in collagen samples modified by increasing light duration at laboratory temp., A is value of fresh collagen solution measured after the reaction with TNBS.*

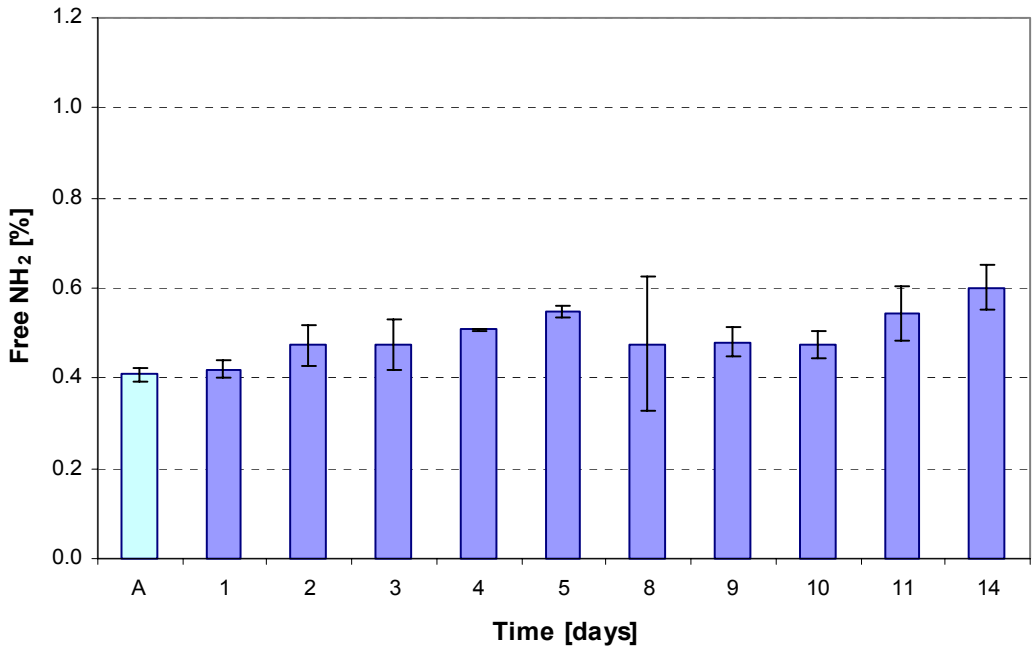


Fig. 21 *Content of free amino groups in collagen samples modified by increasing light duration at laboratory temp. A is value of fresh collagen solution measured after the reaction with ninhydrine.*

4.1.4 Influence of pH adjustment

The amount of free amino groups obtained from the reaction of samples modified by different value of pH (set E) samples with TNBS rising from 2.8 % of the unmodified collagen to 4.1 % of the sample with pH 5. For samples with pH values of 6, 7 and 8 a decrease of free amino groups up to 2.1 % was observed (Tab. A4 in appendix, Fig. 22).

However, the values of free amino groups measured after the reaction with ninhydrin showed an opposite trend compared with TNBS. Samples with lower pH contained much smaller quantity of free amino groups (average 1.2 %) than the samples with pH 6, 7 and 8 of about 5.4 %, (tab.A 4 in appendix, Fig. 23).

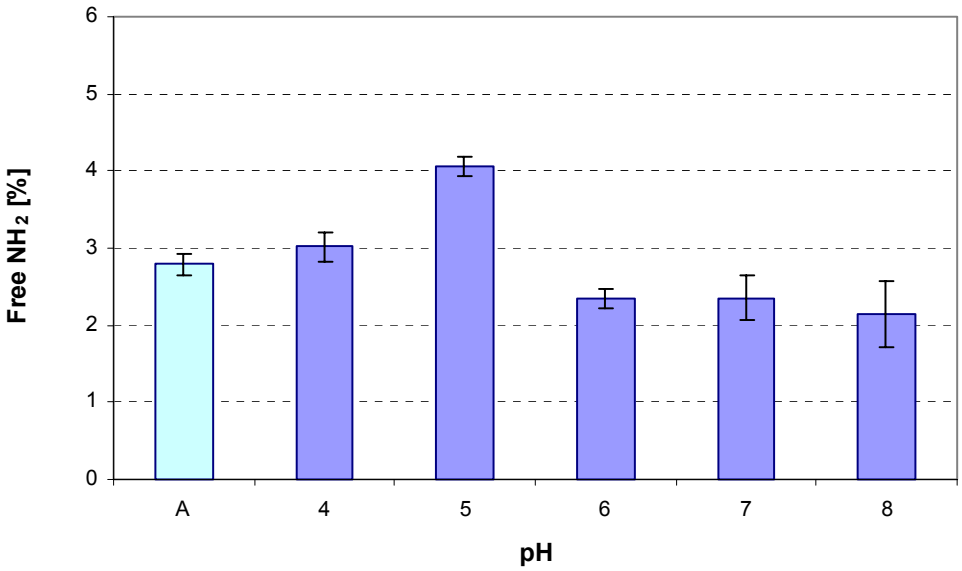


Fig. 22 *Content of free amino groups in collagen samples modified by pH adjusting at laboratory temp., A is value of collagen solution with unmodified pH (3.15), measured after the reaction with TNBS.*

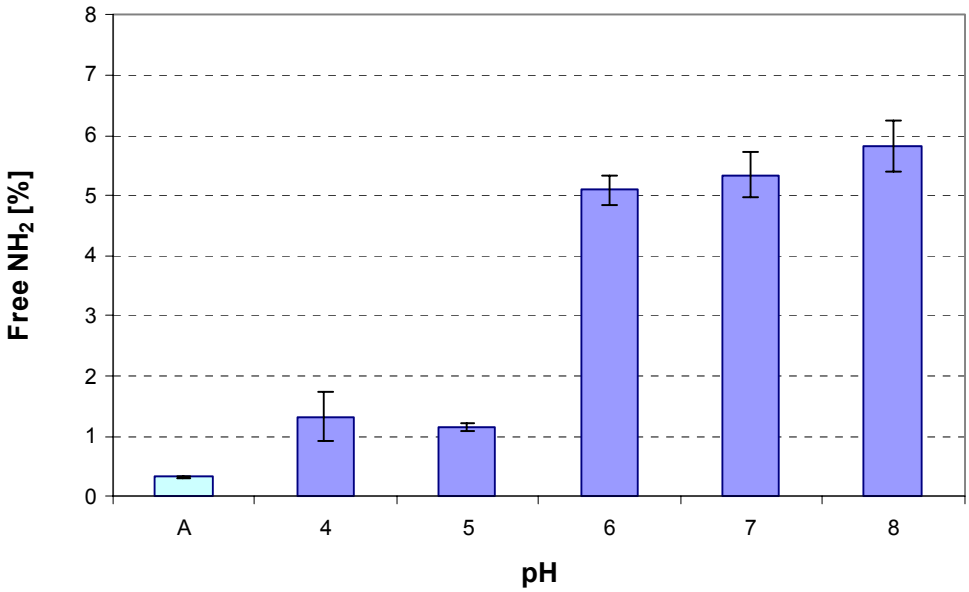


Fig. 23 *Content of free amino groups in collagen samples modified by pH adjusting at laboratory temp., A is value of collagen solution with unmodified pH (3.15) measured after the reaction with ninhydrine.*

4.2 FT-IR spectroscopy

4.2.1 Tablets

Free amino groups present in collagenous sample may react with a suitable reagent to form compounds containing functional groups. The amount can be determined on the basis of the absorbation band. In other words, with adding known quantity of reagent, the amount of free amino group can be assessed. Here, 2,4,6-trinitrobenzensulfonic acid (TNBS) was chosen as the reagent. TNBS reacts with amino group of collagen forming trinitrophenylamine (Fig. **13**). Trinitrophenylamine contains two functional groups absorbing in the infrared region, both aromatic ring (3050 - 2950 cm^{-1}) and nitro group (1550 and 1350 cm^{-1}). These functional groups are not involved in collagen samples, therefore, this groups can be used to determine the quantity of amino groups, which react with TNBS. When compared infrared spectra of pure collagen and collagen after the reaction with TNBS significant changes are evident (Fig. 24).

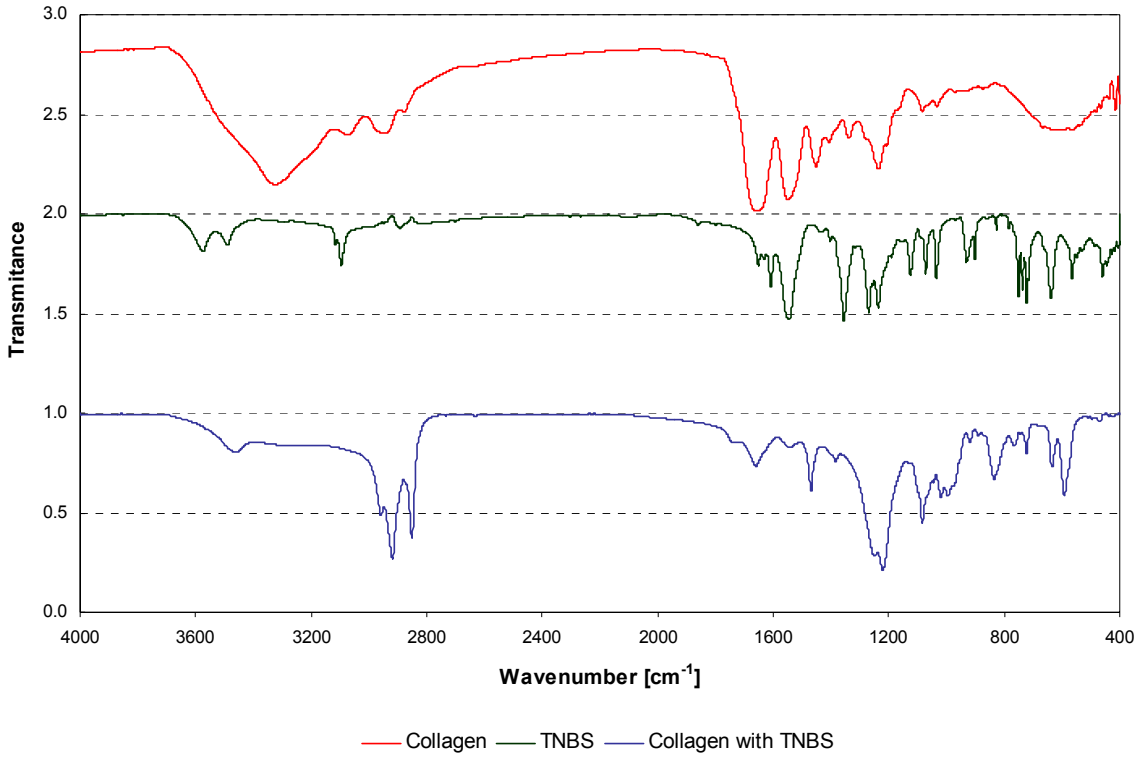


Fig. 24 *Infrared spectra of collagen, TNBS and collagen after reaction with TNBS.*

In the spectrum of pure collagen bands corresponding to amide bonds (1480 - 1750 cm^{-1}) are very intensive. However, in the spectrum of collagen modified with TNBS the amide characteristic peak disappeared and newly some bands were emerged. These bands fit into valency vibration of C-H bonds in saturated systems (under 3000 cm^{-1}), which correspond to the degradation of collagen by splitting the peptide bonds resulting from the reaction of collagen samples with TNBS. Bands characteristic for TNBS (aromatic ring and nitro group), which should be used to determine the quantity of collagen amino groups reacted with TNBS, however, seemed to be hardly interpreted. Therefore, infrared spectra of collagen in the form of films were used to evaluate the quantity of free amino groups. Selected infrared spectra of collagen tablets after the reaction with TNBS are listed in the appendix (appendix A 5 - A 9).

4.2.2 Films

Infrared spectrum of collagen contains several characteristic bands - band of bond of N-H (amide A), band of amide I, band of amide II and band of amide III. The band corresponding to the vibration of N-H is located in the 3400 - 3440 cm^{-1}, however, owing to the hydrogen binding presented in collagen caused moving the N-H band into the area of 3300 cm^{-1} [56].

As for our collagen samples in the form of film prepared from 0.2 % solution, characteristic bands were interpreted on the basis of literature [57]. Band of amide I was found to be located between 1590 - 1720 cm^{-1}, amide II band is in region of wavenumber at 1520 - 1620 cm^{-1} and

band of amide III is located in wavenumber of 1130 - 1300 cm^{-1} (Fig. 25). Bands occuring at 1645 - 1657 cm^{-1} corespond to random coils and the band at 1660 cm^{-1} fit into the triple helix. Area and location of individual peak is changed according to the changes in the structure of collagen. Fibril formation, which increases intermolecular interactions in collagen, is associated with broadening and slight shift to lower wavenumber of the amide A, increase in intensity and slight shift to lower wavenumber of amide III peak and band broadening and shift of amide I peak to lower wavenumber. Denaturation of collagen, on the other hand, is manifested by reduction in intensity of band of amide A, amide I, amide II and amide III and decrease of amide I components in wavenumber of 1630 cm^{-1} and 1660 cm^{-1} [57].

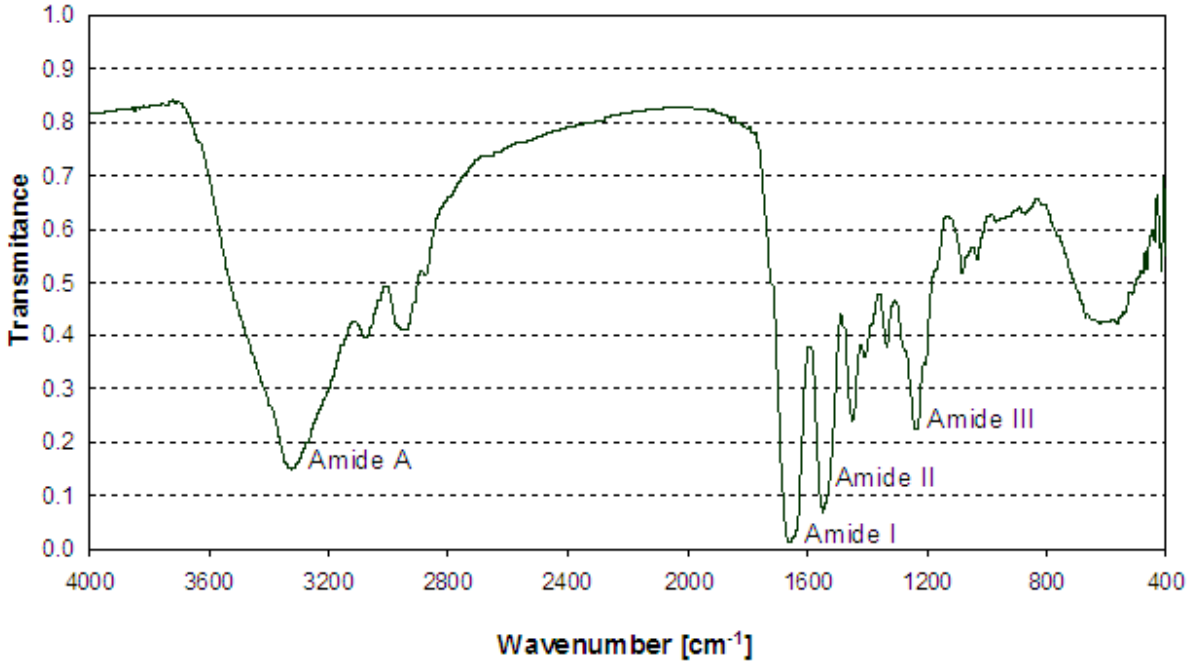


Fig. 25 *Characterization bands in collagen infrared spectrum.*

All these bands reflect the secondary structure of the sample, therefore to evaluate the structure of samples the amide I band, which is used very often for the proteins secondary structure characterization, was used. For better observing changes in the collagen structure, the amid I band (1590 - 1720 cm^{-1}) was deconvulated (splitted) resulting in four separated peaks - peak 1, peak 2, peak 3 and peak 4 having the maximum at 1690 cm^{-1}, 1665 cm^{-1}, 1650 cm^{-1} and 1630 cm^{-1}, respectively (Fig. 26).

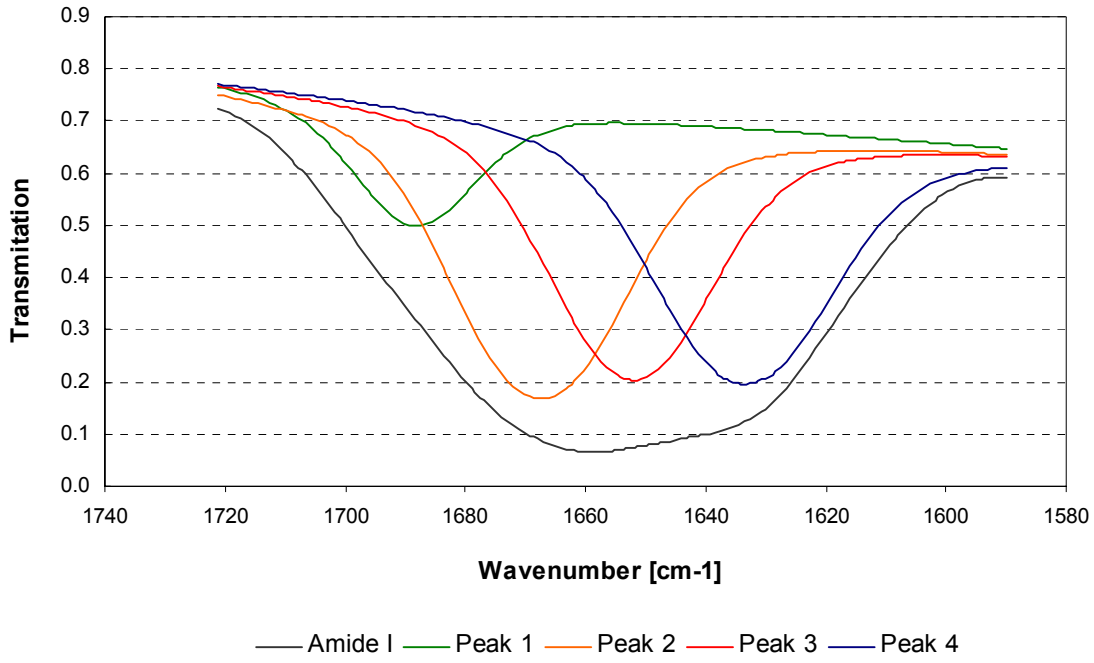


Fig. 26 *Deconvulation of amid I.*

With a view of interpreting the changes occurred during the modification of samples, peaks formed by deconvulation of amide I and their ratio were used. When compared deconvulated peaks of amid I for samples B6, B7, B8 and B10, which were modified by different time and intenzity of disintegration at lab. temperature, a several trends can be observed. Peak 1 as well as peak 4 remained almost unchanged in all samples (Fig. 27, grey and blue columns). For that reason, these peaks were not taken into account for evaluation. Due to large amounts of data only deconvulation of amide I peak for samples B6 - B10 are included in appendix.

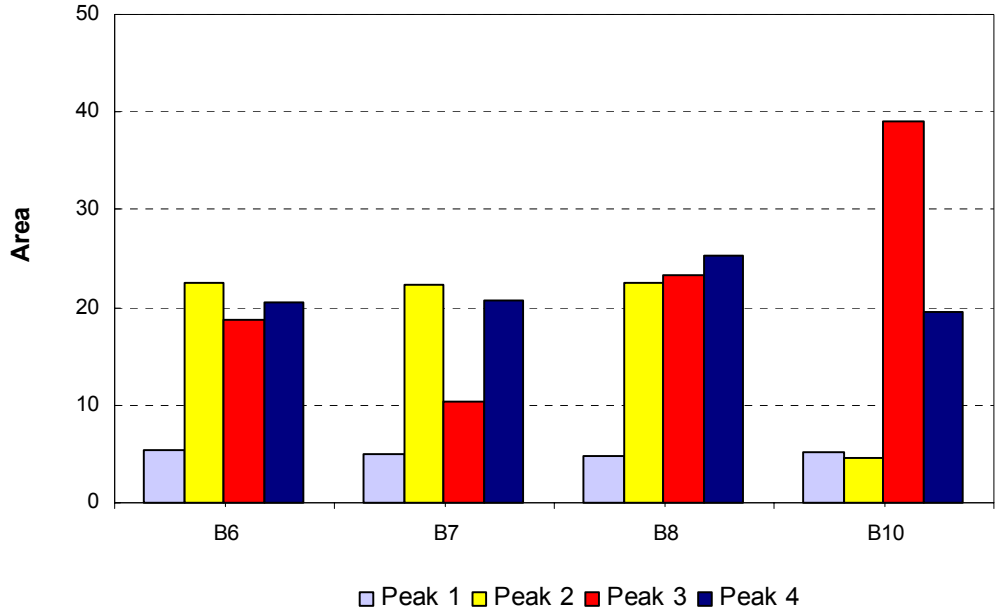


Fig. 27 *Comparison of peaks after deconvulation of amid I peak for samples B6, B7, B8 and B10. Samples were disintegred by different time and intenzity at lab. temperature. Sample B6 was disintegred for 1 min at 6 000 rpm, sample B7 was disintegred for 2 min at 10 000 rpm, sample B8 was disintegred for 3 min at 14 000 rpm and sample B10 was disintegred for 5 min at 14 000 rpm.*

4.2.2.1 Influence of intensity and and time of disintegration

When comparing the areas of each peak of amide I in the samples disintegrated for 1, 2, 3 and 5 min (samples at 4 mins were not characterized), there is the apparent decrease in peak 2 area with increasing temperature and time of disintegration, except for the time of 5 min at 4 °C (Fig. 28). Due to the high amount of measured samples, the films disintegrated for 2, 3 and 5 min at 30 °C were not evaluated. In contrast, the areas of peak 3 increase with raising temperature and time of disintegration (Fig. 29). From area ratio of peak 2 to peak 3 (Fig. 30), it is clear that structure of random coil increased with temperature and intensity of disintegration at the loss of triple helical structure.

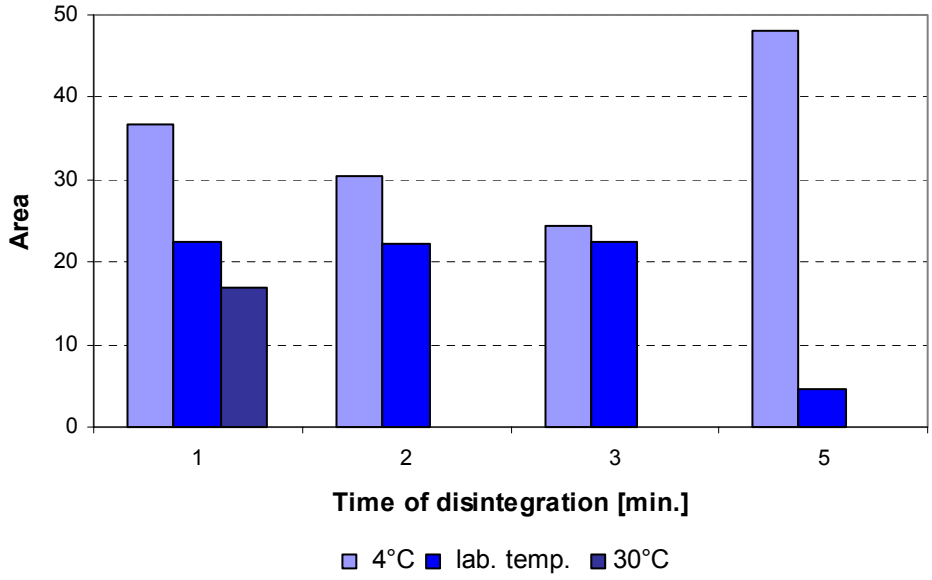


Fig. 28 *Areas of peak 2 for samples of set B, which were modified by time and temperature of disintegration.*

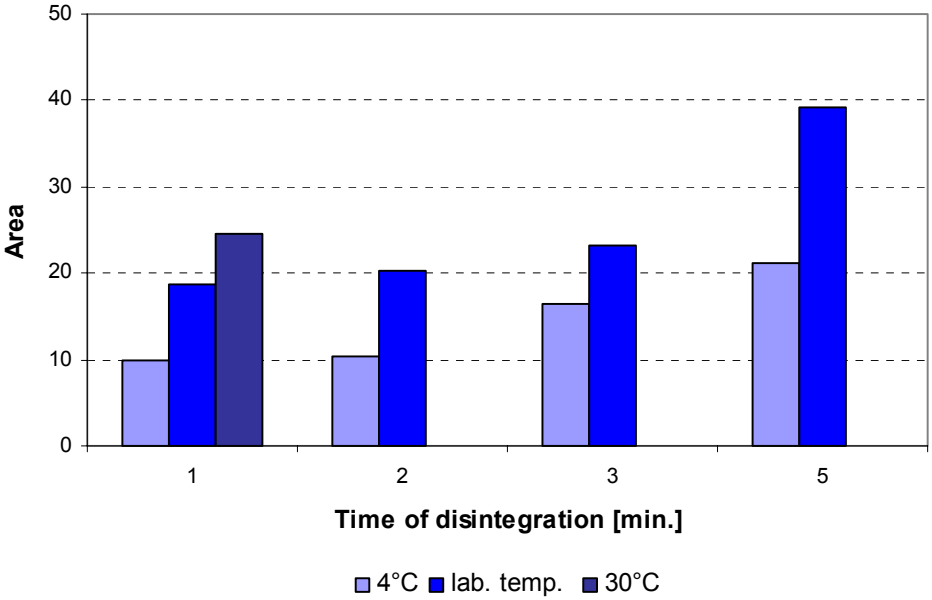


Fig. 29 *Areas of peak 3 for samples of set B, which were modified by time and temperature of disintegration.*

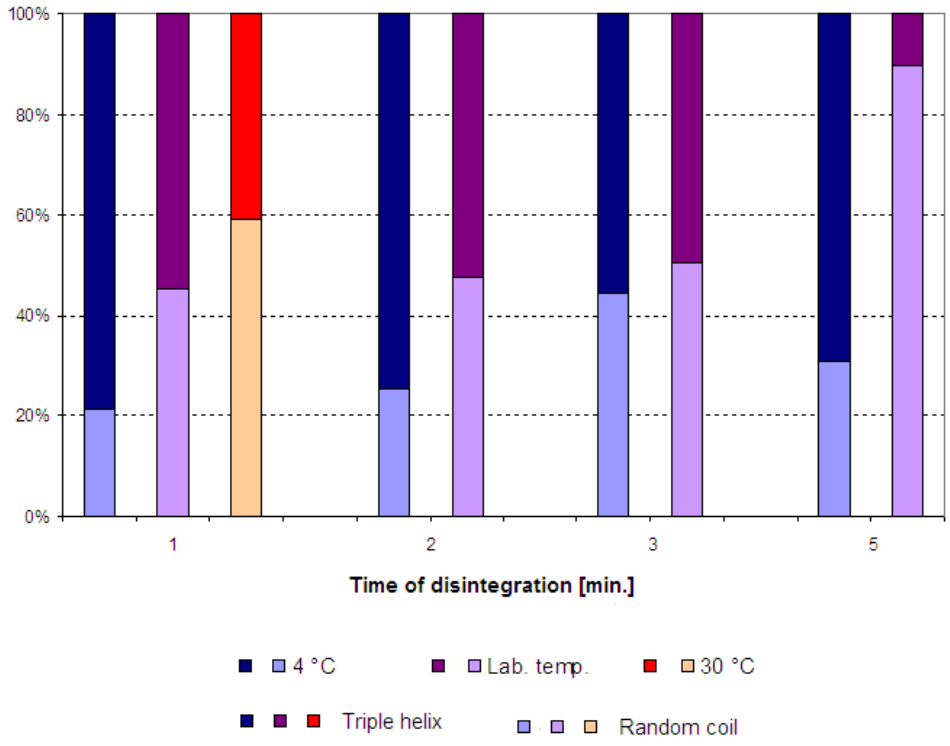


Fig. 30 *Ratio of random coil and triple helix in samples of set B, which were modified by time and temperature of disintegration.*

4.2.2.2 Influence of time and temperature of preparation

When comparing the areas of samples that were modified by various times taking from preparation untill freezing at different temperatures (samples C11 and C14 were not characterized) in dark, decreasing trend of peak 2 was observed (Fig. 31). By contrast, the areas of peak 3 show an increasing trend (Fig. 32). Ratio of areas of peak 2 to peak 3 show an increase of random coil structure and decrease of triple helix (Fig. 33), in most cases.

The temperature and the time lag between preparation of sample and freezing of sample has a negative effect on collagen structure.

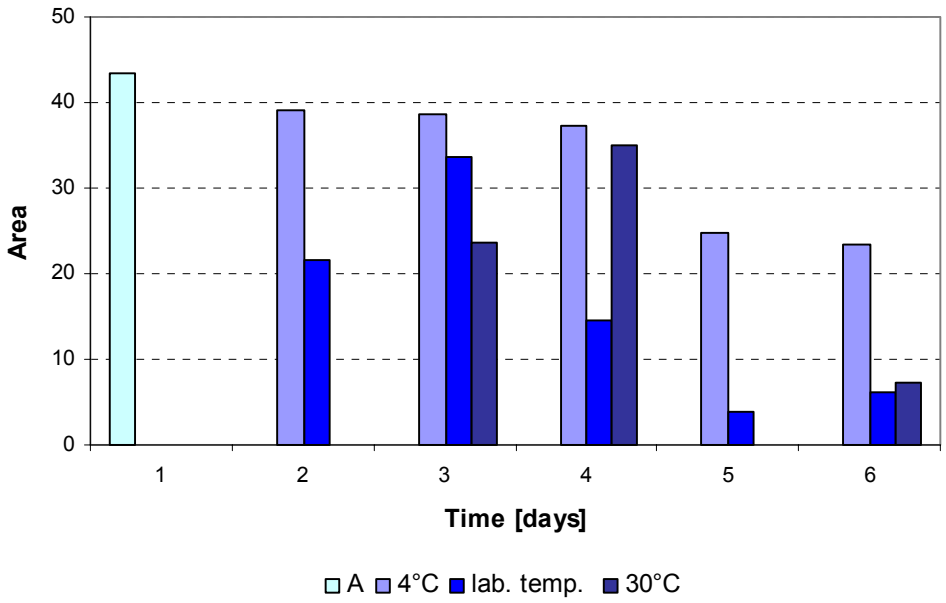


Fig. 31 *Areas of peak 2 for samples modified by different processing time proceeded from the preparation till freezing upon three different temperatures (4 °C, lab. temp. and 30 °C) in dark. Samples were disintegrated for 3 min at 14 000 rpm. A is value of fresh prepared collagen.*

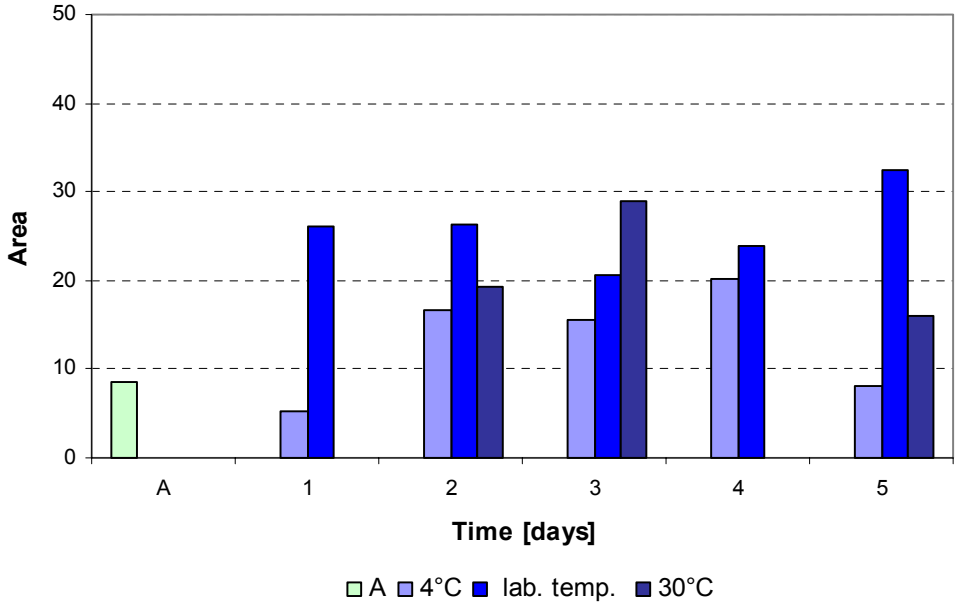


Fig. 32 *Areas of peak 3 for samples modified by different processing time proceeded from the preparation till freezing upon three different temperatures (4 °C, lab. temp. and 30 °C) in dark. Samples were disintegrated for 3 min at 14 000 rpm. A is value of fresh prepared collagen.*

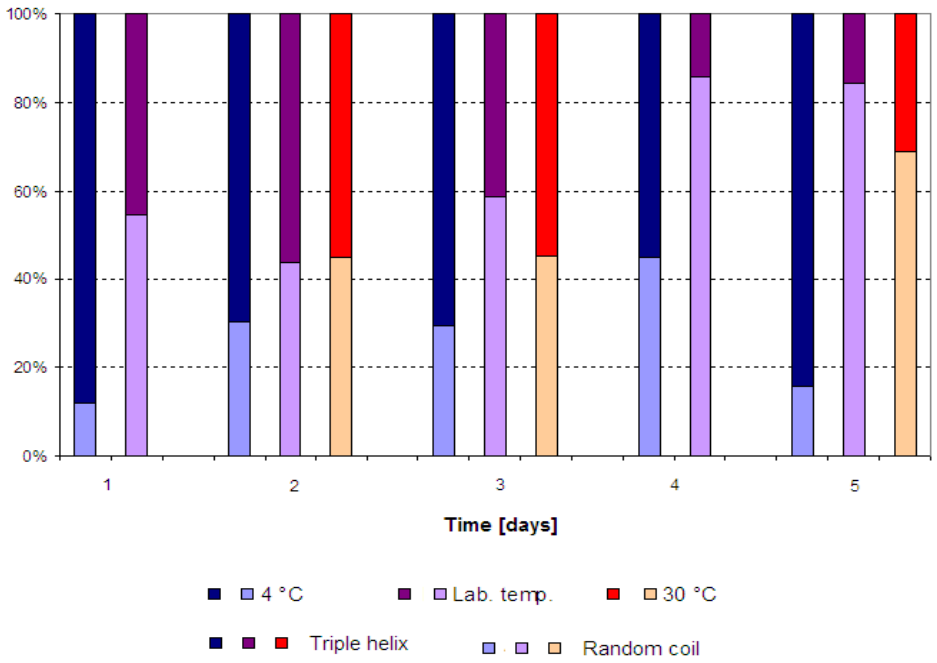


Fig. 33 *Ratio of random coil and triple helix in samples of set C, which were modified by different processing time and different temperatures.*

4.2.2.3 Influence of light duration

The areas of peak 2 obtained by analysis of samples modified by different processing time proceeded from the preparation till freezing at light (set D) have irregular but decreasing trend (Fig. 34).

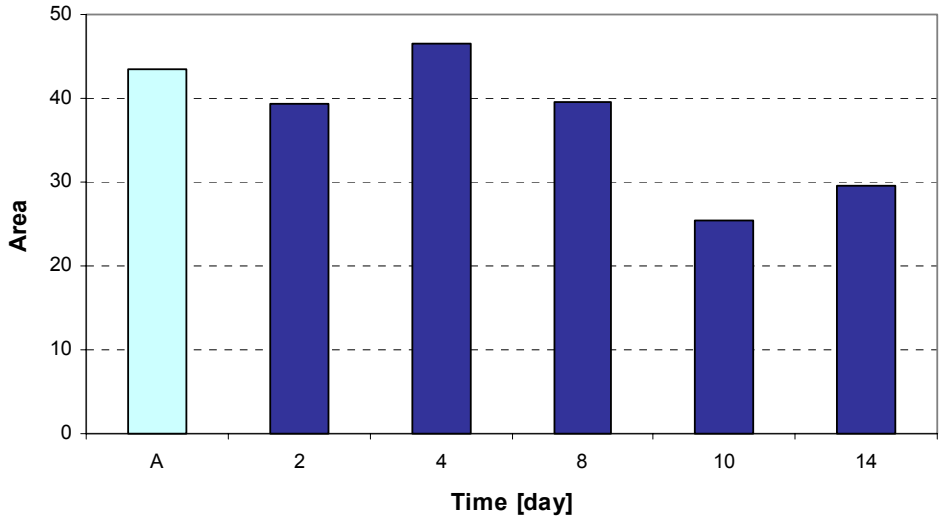


Fig. 34 *Areas of peak 2 for samples of set D, which were modified by different time from preparation till freezing at light. Samples were disintegrated for 3 min at 14 000 rpm A is value of fresh prepared collagen solution.*

Areas of peak 3 show, in comparison with freshly prepared solution of collagen, slight decrease within first days of the light duration, which may be caused by partial cross-linking. With time longer tan 4 days, however, a very significant increase in the area of peak 3 (Fig. 35) occured. The ratios between the two peaks correspond to significant increase in random coil structure and the loss of triple helix structure (Fig. 36). Therefore, during standing of collagen samples on the light a gradual distortion in the structure of collagen occurs.

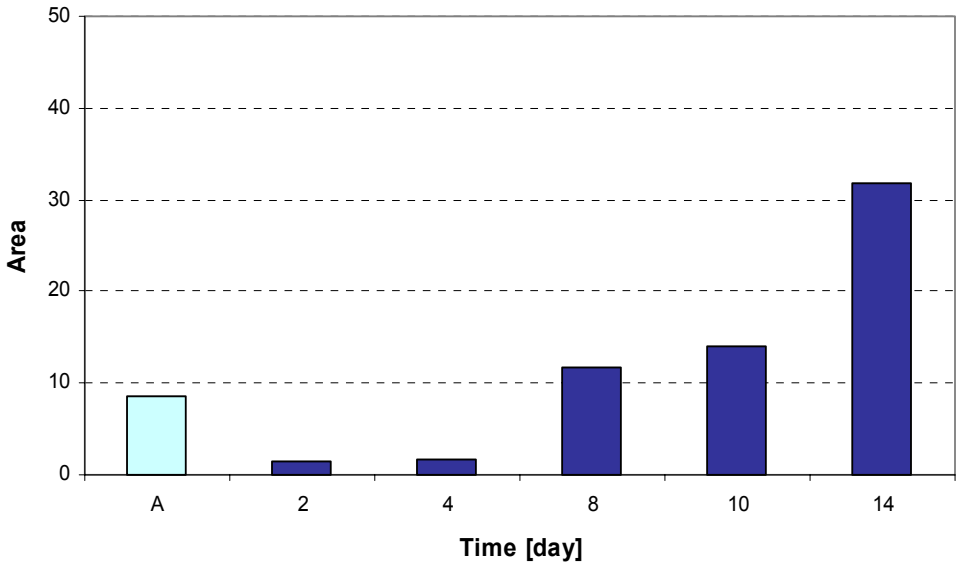


Fig. 35 *Areas of peak 3 for samples of set D, which were modified by different time from preparation till freezing at light. Samples were disintegrated for 3 min at 14 000 rpm. A is value of fresh prepared collagen solution.*

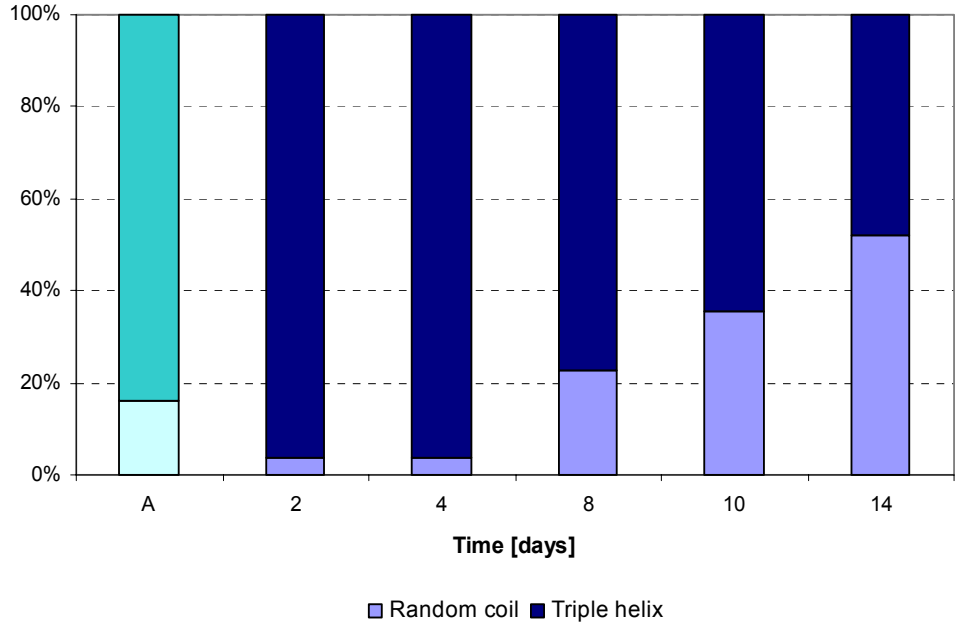


Fig. 36 *Ratio of random coil and triple helix in samples, which were modified by different processing time at light. A is values of fresh prepared collagen.*

4.2.2.4 Influence of pH adjustment

Areas of peak 2 and peak 3 obtained by deconvulaton of amid I band for samples modified by pH adjustment (set E) are very irregular. In the case of peak 2, there is an decrease in area at pH of 4 and 5 in comparison with set A. The area reaches a maximum at pH equal to 6 from where the values decreases (Fig. 37). The areas of peak 3 are very small for samples of pH 4, 6 and 8 and high at pH 5 and 7 (Fig. 38). Anomalies are probably caused by nonhomogeneous films which resulted from the coagulation of collagen solution at higher values of pH (Fig. 40 and Fig. 41).

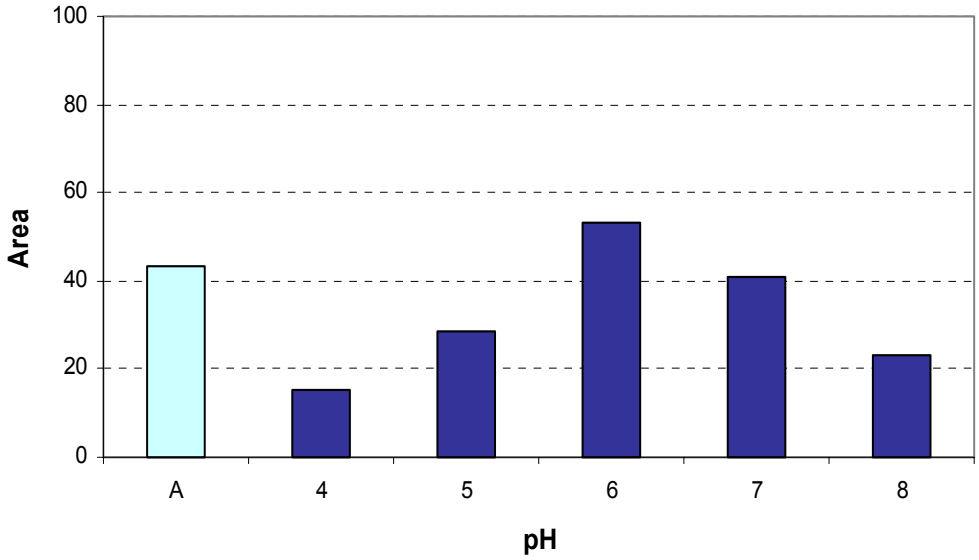


Fig. 37 *Areas of peak 2 for samples modified by different values of pH. Samples were disintegrated for 3 min at 14 000 rpm A is value for collagen is natural pH.*

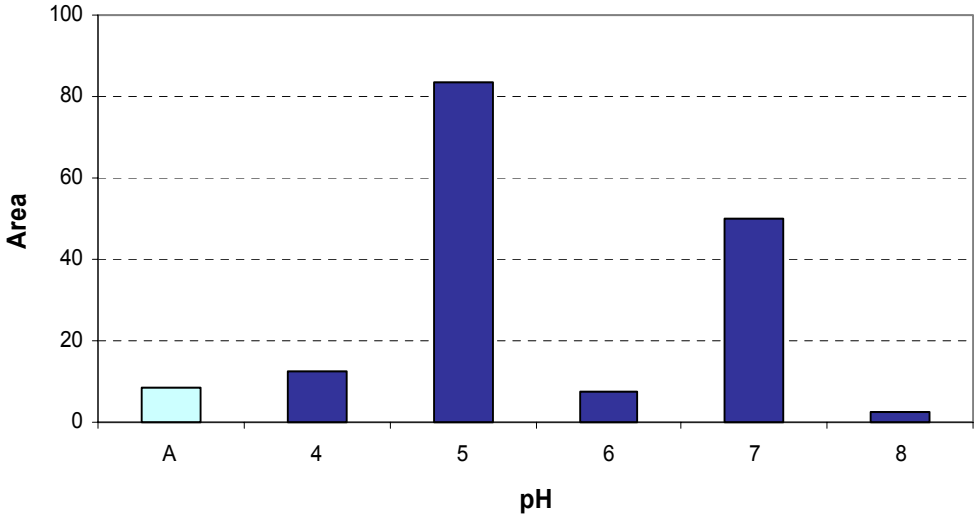


Fig. 38 *Areas of peak 3 for samples modified by different values of pH. Samples were disintegrated for 3 min at 14 000 rpm A is value of collagen with natural pH.*

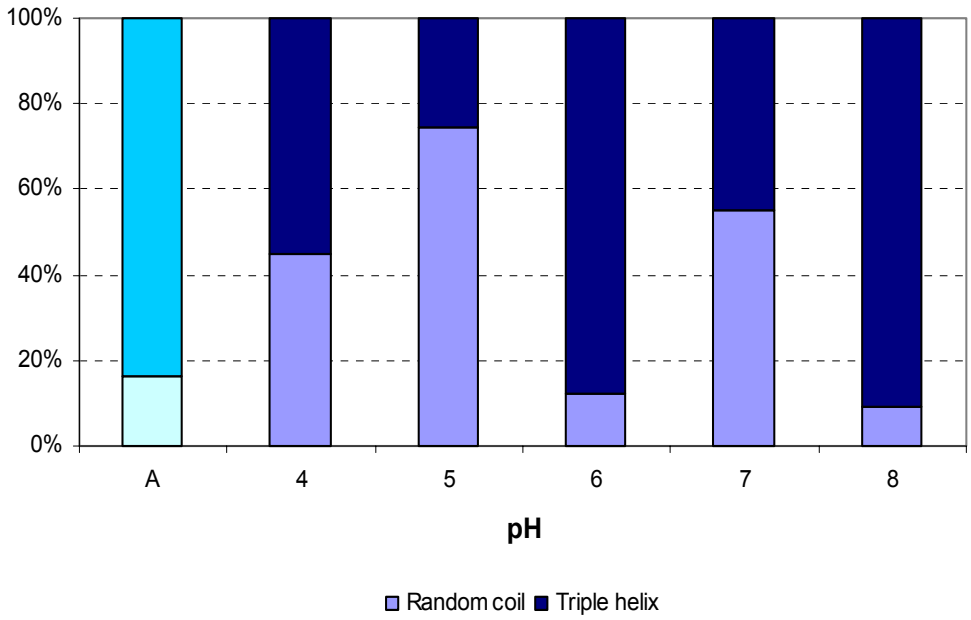


Fig. 39 *Ratio of random coil and triple helix in samples with different value of pH. A is values of fresh prepared collagen.*

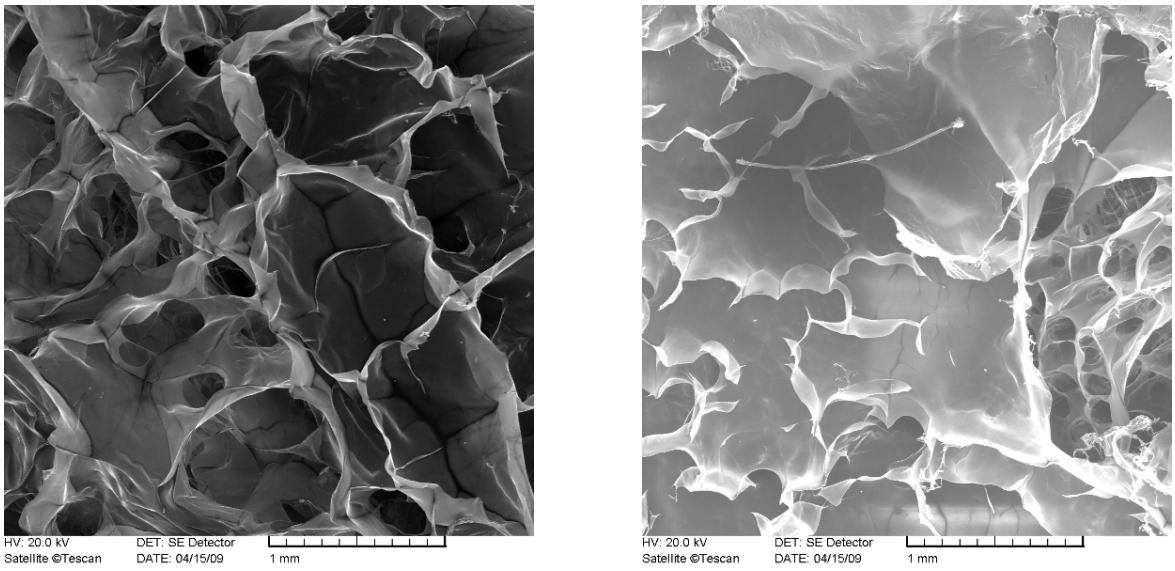
Fig. 40 *Water solution of collagen at concentration of 0.2 wt%. A is collagen with pH = 3.15, E1 is collagen with pH = 4 and E2 is collagen with pH = 5.*

Fig. 41 *Water solution of collagen at concentration of 0.2 wt%. E3 is collagen with pH = 6, E4 is collagen with pH = 7 and E5 is collagen with pH = 8.*

4.3 Morphology

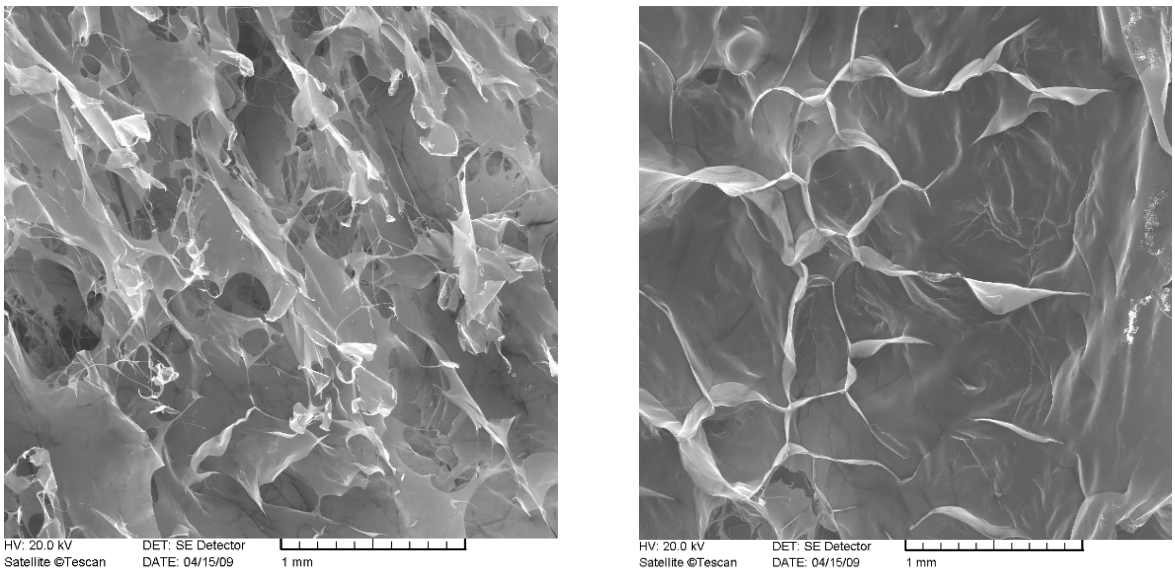
Because of the significant structural changes observed in the preparation of samples treated by different processing time proceeded from the preparation till freezing at light (set D) and samples treated by different value of pH (set E), this samples were observed using SEM. Due to the very different results from SEM pictures assessing, porosity and pore size were not determined, only samples morphology was compared.

Freshly prepared collagen samples show regular porous structure. This structure is still visible at the sample, which was left at laboratory temperature on the light for 2 days (Fig. 42a). However, during the intervening time from preparation untill freezing (e.g.up to 10 days), increasing the pore size and ruining the porous structure occured (Fig. 42b). The most considerable change in the structure is evident for collagen samples with pH adjustment. Pores size increase with increasing pH but the change in sample morphology is evident at pH 5 (Fig. 43), at which the collagen become separated from the solution. Further increasing in the pH value cases the irregularity in the morphology and very different pore size distribution as show samples with pH 6, 7 and 8 at Fig. 44a, Fig. 44b and Fig. 45, respectively.



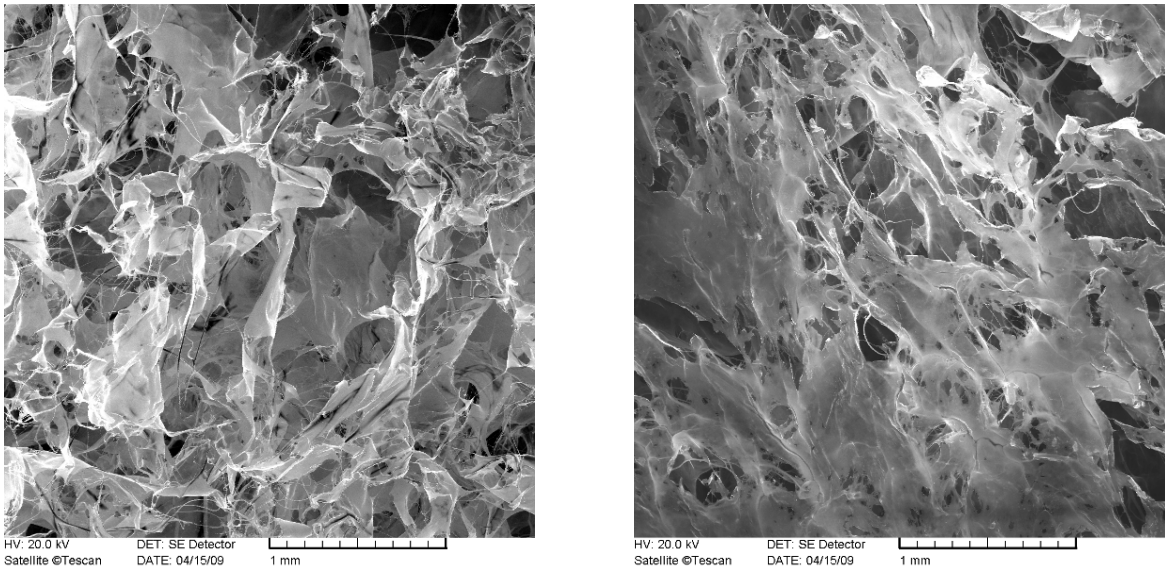
a) *b)*

Fig. 42 *Morphology of collagen lyophilizate of set D observed by SEM. a) collagen at concentration of 0.2 wt% after 2 days, b) collagen at concentration of 0.2 wt% after 10 days standing on the light.*



a) *b)*

Fig. 43 *Morphology of collagen lyophilizate observed by SEM. a) collagen at concentration of 0.2 wt% with pH = 4, b) collagen at concentration of 0.2 wt% with pH = 5.*



a) *b)*

Fig. 44 *Morphology of collagen lyophilizate observed by SEM. a) collagen at concentration of 0.2 wt% with pH = 6, b) collagen at concentration of 0.2 wt% with pH = 7.*

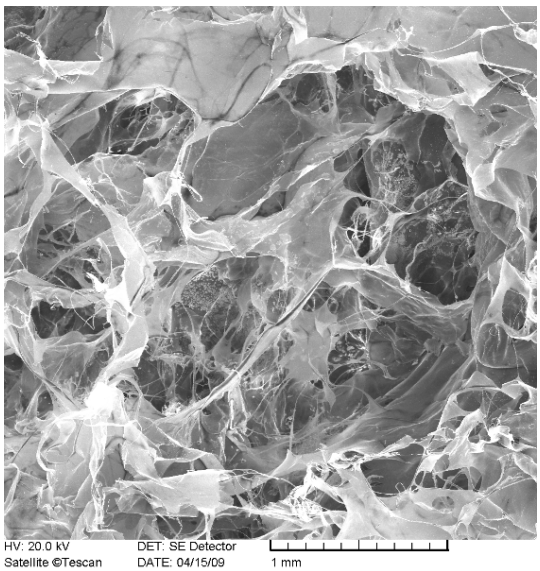


Fig. 45 *Morphology of collagen lyophilizate observed by SEM. Collagen at concentration of 0.2 wt% with pH = 8.*

5 CONSLUSION

In this work, modified water solutions of the bovine collagen type I were analyzed with a view of finding out the changes in collagen structure prepared at different physical conditions. Collagen samples were prepared by varying conditions including changing the intensity (from 1 min at 6 000 rmp to 5 min at 14 000 rpm) and temperature (4 °C, lab. temp. and 30 °C) of collagen disintegration, time (from 1 day up to 5 days) and temperature (4 °C, lab. temp. and 30 °C) of preparation, light duration (from 1 day up to 14 days) and pH (from 4 to 8). Prepared modified samples were compared with unmodified collagen sample characteristics.

In the case of UV-VIS analysis of samples modified by varying intensity and duration of disintegration significant changes of the quantity of free amino groups were not found, only increasing the temperature up to 30 °C showed sligh decrease in the amino groups' content. Regarding the samples modified by different processing time proceeded from the preparation till freezing upon three different temperatures, amount of free amino groups increases with extending time and growing temperature of preparation. The structure of collagen was significantly negatively affected by increasing temperature and the delay between sample preparation and freezing before lyophilization. For samples modified by different processing time proceeded from the preparation till freezing, which were left at laboratory temperature on the light an increase in the amount of free amino groups with time is evident. When leaving the samples on the light, slight cross-links arise within first days but it is not accompanied by loss of the amount of free amino groups. Within longer collagen sample standing on the light very significant changes in the samples' consistency were observed and sample became poorly liquid. For samples modified by pH adjustment value obtained after reaction with TNBS and ninhydrin show an opposite trend, probably due to differences in response mechanisms, where an increase of pH leads to significant increase in the values obtained by reaction with ninhydrin. Collagen samples were stable and homogeneous up to the pH = 5 but since the collagen started separing and precipitating, which have a negative impact on the application of collagen samples.

To evaluate changes in the collagen structure the deconvulation of the amide I group having characteristic band between 1590 - 1720 cm^{-1} in the infrared spectrum of collagen was used. By evaluating the spectra obtained from FT-IR it is clear that structure of random coil increases with temperature and intensity of disintegration at the loss of triple helical structure. The increasing temperature and the time lag between preparation of sample and freezing of sample have negative effect on collagen structure. Also during standing of collagen samples on the light a gradual distortion of the collagen structure occurs. Values obtained by infrared spectroscopy of samples with adjusting pH were very irregular. Anomalies are probably caused by nonhomogenous film, which resulted from the coagulation of collagen solution at higher values of pH.

In order to prepare collagen samples with minimal changes in physico-chemical properties, samples should be prepared freshly, disintegrated for about 4 min at 14 000 rpm at 4 °C without pH adjusting and immediately freezed with no standing on the light.

The study of morphology by SEM on the collagen lyophilized sponges were very important to evaluate the potential capacity in cell-sponge interactions. SEM analysis confirmed that increasing the light duration and the value of pH over 5 causes ruining the porous structure and increasing of pores' size. Observed changes may negatively affect the applicability of collagen sponges e.g. in tissue engineering utilizing seeding cells.

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7 LIST OF APPENDIXES

7.1 UV-VIS spectroscopy

7.2 FT-IR spectroscopy

8 APENDIXES

A 1 *Content of free amino groups in samples of set B.*

Sample	After reaction with TNBS		After reaction of ninhydrine	
	Concentration of $NH_2 . 10^3$ [mg/ml]	Free NH_2 [%]	Concentration of $NH_2 . 10^3$ [mg/ml]	Free NH_2 [%]
B1	7.484	3.690	1.057	0.521
B2	7.223	3.561	1.200	0.558
B3	7.629	3.762	1.063	0.524
B4	6.765	3.336	1.095	0.540
B5	7.660	3.777	1.184	0.584
B6	8.057	3.973	1.727	0.851
B7	8.280	4.083	1.617	0.797
B8	9.518	4.693	1.063	0.734
B9	8.571	4.227	1.274	0.628
B10	8.297	3.866	1.420	0.700
B11	1.705	2.256	1.196	0.590
B12	2.627	2.601	1.148	0.566
B13	7.678	3.786	1.235	0.609
B14	4.197	2.070	1.164	0.574
B15	8.673	4.277	1.235	0.609

A 2 *Content of free amino groups in samples of set C.*

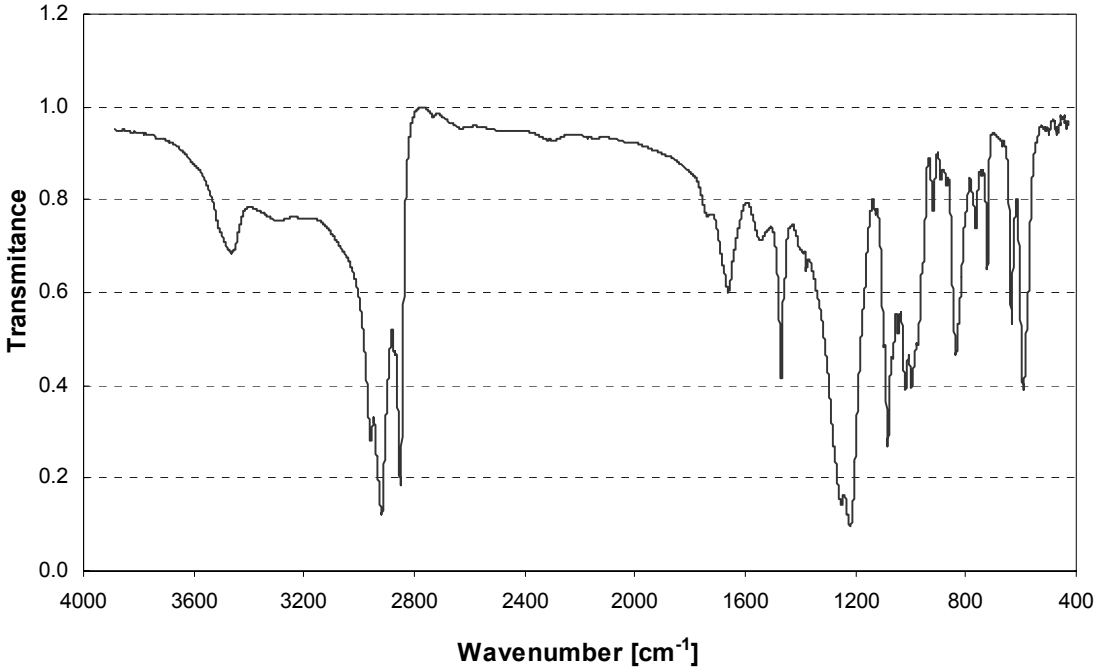
Sample	After reaction with TNBS		After reaction of ninhydrine	
	Concentration of $NH_2 . 10^3$ [mg/ml]	Free NH_2 [%]	Concentration of $NH_2 . 10^3$ [mg/ml]	Free NH_2 [%]
C1	5.414	2.375	1.080	0.532
C2	5.347	2.675	1.154	0.569
C3	6.690	3.215	1.222	0.603
C4	6.843	3.406	1.255	0.619
C5	8.582	3.242	1.229	0.606
C6	4.817	1.802	1.190	0.587
C7	5.425	2.637	1.222	0.603
C8	6.520	3.299	1.148	0.564
C9	6.907	3.374	1.112	0.548
C10	6.575	4.232	1.144	0.564
C11	4.618	2.277	1.267	0.625
C12	7.714	4.490	1.297	0.639
C13	8.789	4.334	1.203	0.593
C14	8.819	4.349	1.303	0.643
C15	10.146	5.003	1.154	0.569

A 3 *Content of free amino groups in samples of set D.*

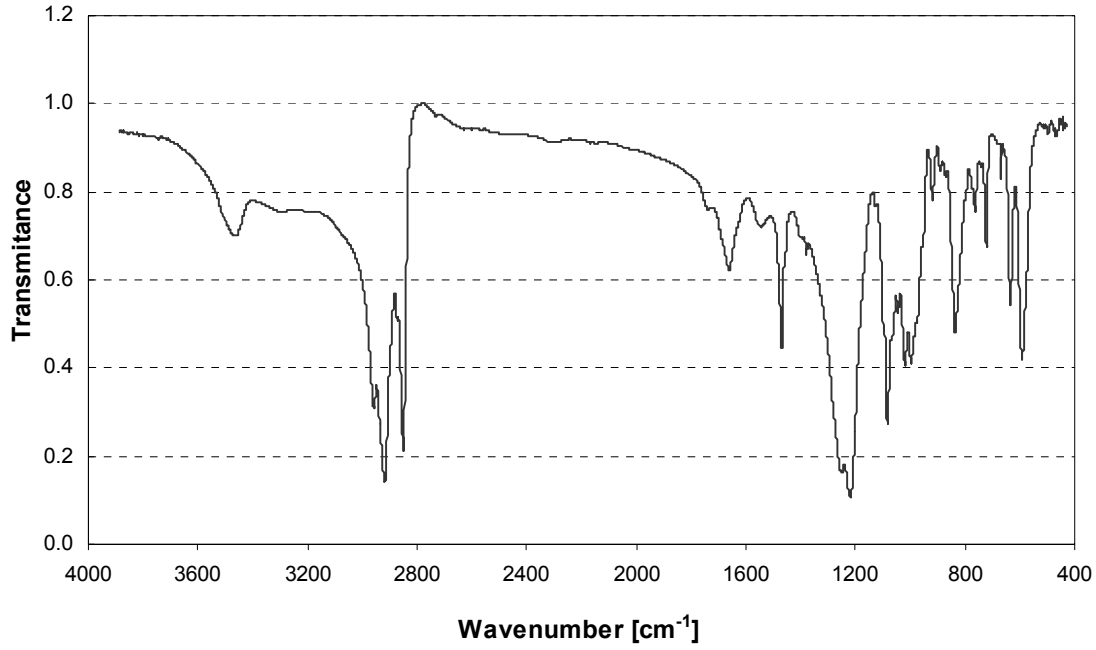
Sample	After reaction with TNBS		After reaction of ninhydrine	
	Concentration of $NH_2 . 10^3$ [mg/ml]	Free NH_2 [%]	Concentration of $NH_2 . 10^3$ [mg/ml]	Free NH_2 [%]
D1	6.770	3.338	0.850	0.419
D2	7.081	3.491	0.959	0.473
D3	7.552	3.724	0.963	0.475
D4	8.717	4.298	1.029	0.507
D5	7.843	3.867	1.094	0.549
D6	7.605	3.748	0.966	0.476
D7	7.522	5.022	0.974	0.480
D8	8.803	4.341	0.963	0.475
D9	11.092	5.469	1.106	0.545
D10	8.333	5.934	1.221	0.602

A 4 *Content of free amino groups in samples of set E.*

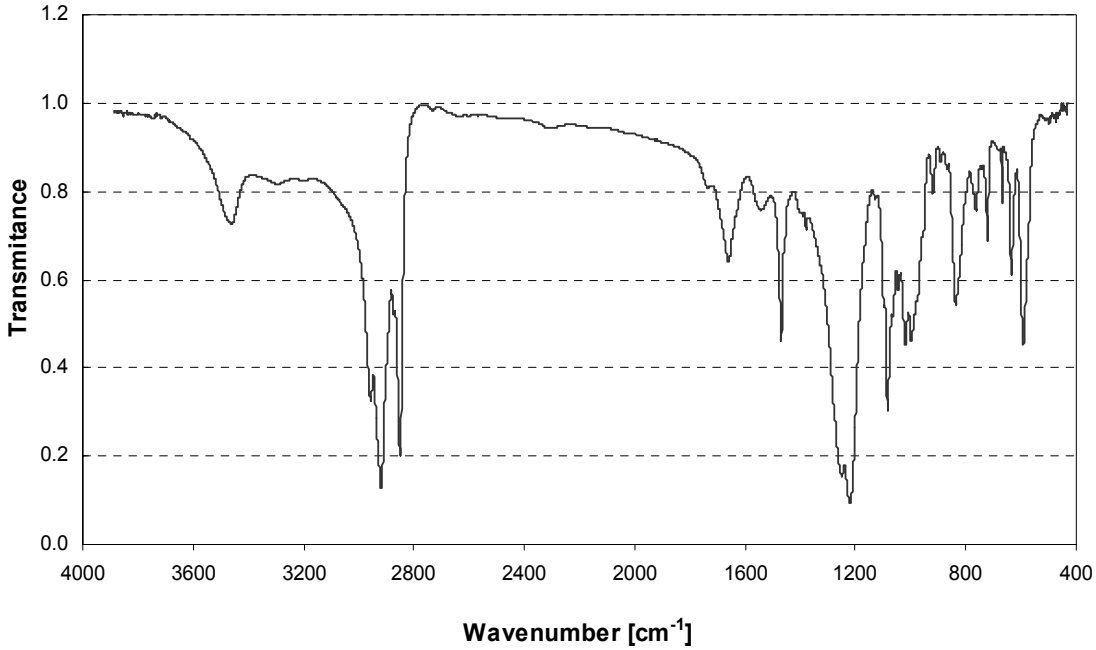
Sample	After reaction with TNBS		After reaction of ninhydrine	
	Concentration of $NH_2 . 10^3$ [mg/ml]	Free NH_2 [%]	Concentration of $NH_2 . 10^3$ [mg/ml]	Free NH_2 [%]
A	5.662	2.792	0.668	0.330
E1	6.124	3.020	2.686	1.324
E2	8.217	4.052	2.339	1.154
E3	4.740	2.337	10.333	5.095
E4	4.764	2.349	10.855	5.353
E5	4.351	2.146	11.808	5.822



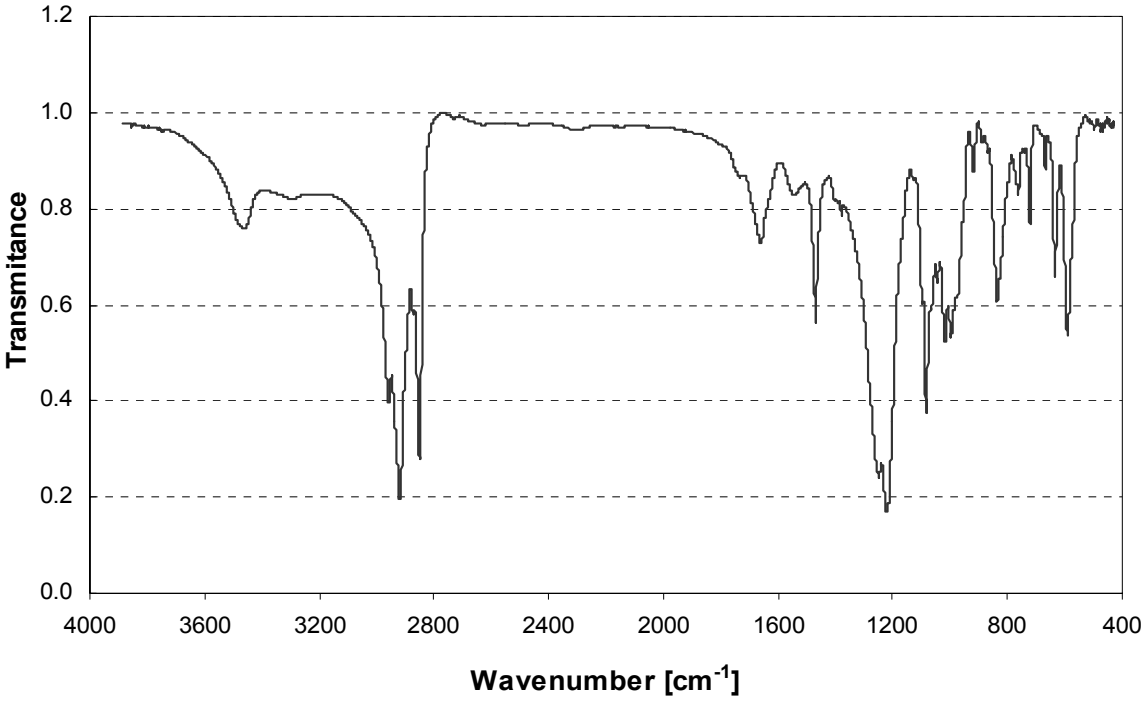
A 5 *Infrared spectrum of tablets prepared from sample C4.*



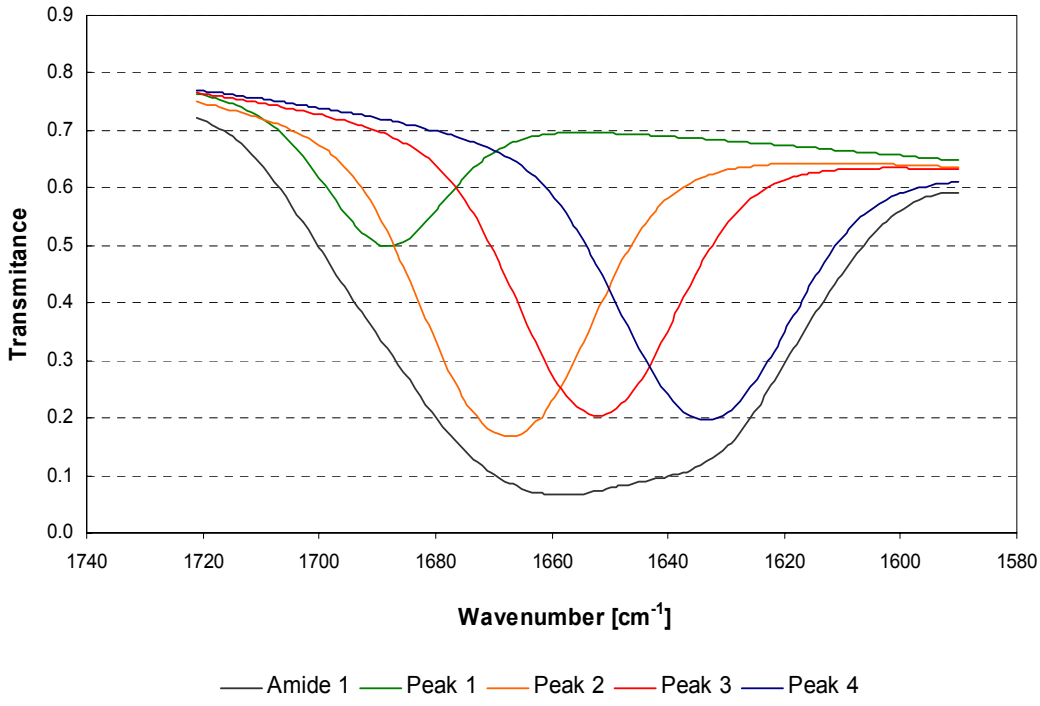
A 6 *Infrared spectrum of tablets prepared from sample D2.*



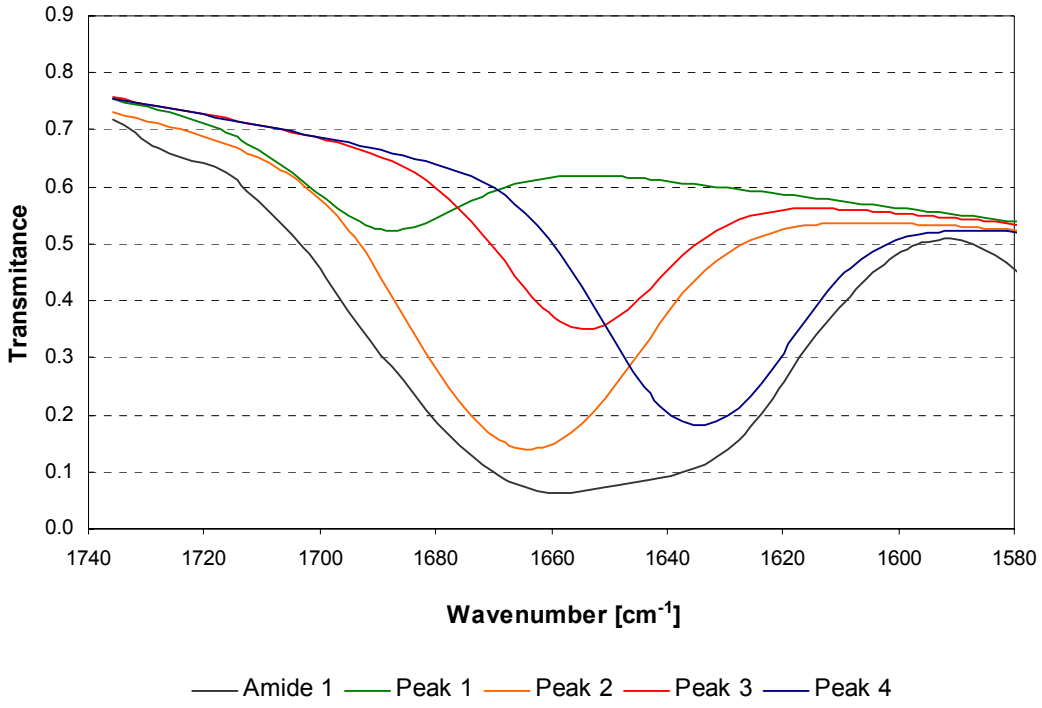
A 7 *Infrared spectrum of tablets prepared from sample D4.*



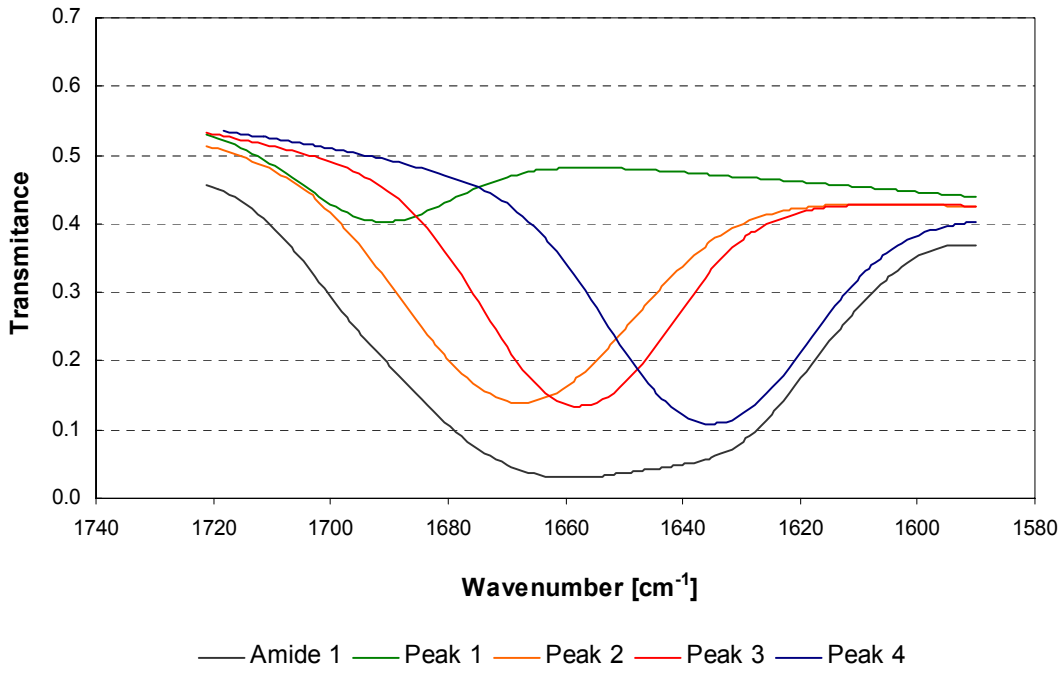
A 8 *Infrared spectrum of tablets prepared from sample D6.*



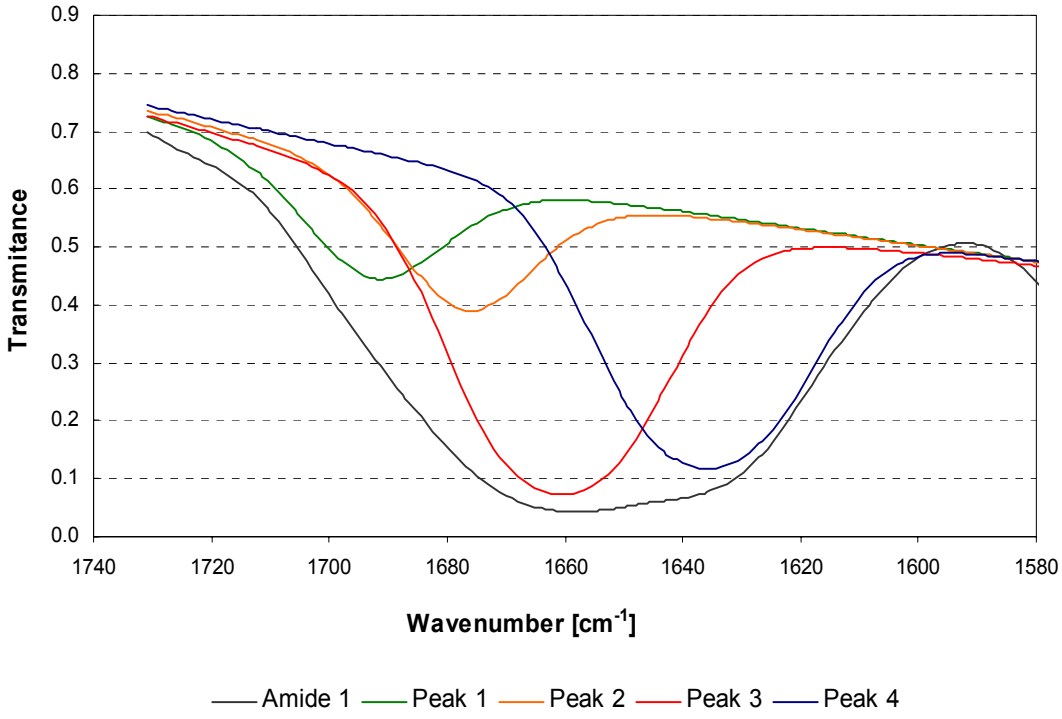
A 9 *Deconvulation of amide I band of sample B6.*



A 10 *Deconvulation of amide I band of sample B7.*



A 11 *Deconvulation of amide I band of sample B8.*



A 12 *Deconvulation of amide I band of sample B10.*